

**Development of Low-Cost Manufacturing Process
For 1,2,4-Butanetriol (BT)**

SBIR Topic No.: N01-073

Contract No.: N00174-01-0025

Phase I Final Report

1 May – 31 October 2001

***Creative Applied Technical Systems, Inc.
8320 Alban Road, Suite 900
Springfield, VA 22150***

Distribution Statement: Approved for public release; Distribution unlimited.

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Summary

The objective of this Phase I project was to demonstrate the feasibility of producing 1,2,4-butanetriol from malic acid via microbial reduction of the carboxylic groups with *Clostridium thermoaceticum*. Unfortunately, this objective was not met. A small amount of 1,2,4-butanetriol was produced. However, the project time was over before the correct process parameters were found to produce 1,2,4-butanetriol in a yield sufficient to evaluate the process. The microbial reduction process parameters are very dependent on the carboxylic acid being reduced, especially for the dicarboxylic acids. The correct combination of pH, reaction time, reaction temperature, microbe concentration, microbe age, reactant concentration, buffer type and concentration, mediator, and pressure must be found. Due to problems with analysis of 1,2,4-butanetriol and malic acid in an aqueous buffer solution and problems with the viability of the original microbes purchased, insufficient time remained on the project to optimize all these parameters.

Two substrates, butyric acid and succinic acid, were used as model compounds for initial process optimization. Reduction of these acids to the corresponding alcohols was accomplished in our laboratory and compared to published literature results. Even with minimal optimization of the experimental parameters, we obtained better yields than those reported in the literature.

Microbial reduction of carboxylic acids is a viable method for making alcohols that are difficult to make by other processes. Since the microbe used for the reduction process also produced acetic acid from sugars in an almost quantitative yield, it is possible for the total process to yield two valuable products. If wastes, *e.g.*, chicken waste or manure, can be utilized as the major component of the growth medium for the microbe, then the process can serve the additional goal of reducing environmental contamination.

Based on our experimental data on *Clostridium thermoaceticum* and reduction of carboxylic acid, preliminary designs for a continuous bench reactor were formulated. This continuous-flow bench-scale reactor includes a recycle fermenter that will produce both microbial cells and acetic acid and a trickling filter reactor for carboxylate reduction.

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1. Objectives

The overall objective of this project was to develop a method to produce high-purity, low-cost 1,2,4-butanetriol (BUT) for use in the synthesis of 1,2,4-butanetrinitrate (BTTN). A second objective was the development of a process for the synthesis of BUT that is environmentally benign. The specific objectives of Phase I of the project were to:

- Demonstrate the feasibility of producing BUT from malic acid via microbial reduction of the carboxylic acid groups.
- Demonstrate that the microbial reduction of the carboxylic acid groups can be accomplished with the anaerobic microorganism, *Clostridium thermoaceticum*.
- Demonstrate that the BUT produced by this method is free of erythritol, threitol and other impurities that could interfere with the thermal stability of BTTN subsequently made from the BUT.
- Optimize process parameters to maximize yield of BUT while minimizing reaction time and production of impurities.

2. Background

BUT is used as the starting material for the production of BTTN, a plasticizer for nitrocellulose used by the military in some minimum smoke propellants. Currently, nitroglycerin is the most widely used plasticizer in these propellants. BTTN has several advantages over nitroglycerin as a propellant plasticizer, *i.e.*, it is less shock sensitive than nitroglycerin, has a lower freezing point, is significantly less volatile, and more thermally stable than nitroglycerin [Piscane, 1982]. However, BTTN has two problems associated with its use as a propellant plasticizer - the high cost of the BUT starting material (60 to 80 times that of glycerin) and the presence of impurities (*e.g.*, erythritol and threitol) in the BUT that are subsequently found in their nitrated forms in the BTTN. Small amounts of erythritol tetranitrate and especially threitol tetranitrate lead to decreased thermal stability of the BTTN plasticizer in the propellant resulting in gas formation and ultimately, in the formation of voids in the propellant [Alley *et al.*, 1986].

Of the many potential methods for synthesis of BUT, the reduction of malic acid appeared to have the most promise for production of a high purity BUT product at a reasonable cost. Malic acid is inexpensive (\$0.80 to \$1.05/lb in less than truck load quantities) and is commercially available from US manufacturers.

2.1 Properties and Reactions of Malic Acid

Malic acid is an optically active dicarboxylic acid having the structures shown in Figure 1. It is manufactured in the United States normally by a high-pressure, high-temperature hydration of maleic acid that in turn is made by vapor phase oxidation of

benzene over a vanadium pentoxide catalyst. The commercial manufacture of malic acid produces the racemic mixture. A typical analysis of the commercial product is presented in Table I. Malic acid is mainly used in the food industry as a flavoring agent and acidulant. However, unlike the other starting materials for the production of BUT, malic acid can be derived from renewable sources. The levorotatory form of malic acid (*l*-malic acid) is found in unripe apples and other fruits. In nature, malic acid can be made by a variety of pathways. It is one of the components of the tricarboxylic acid cycle (TCA) which is the major energy producing metabolic pathway for the oxidation of pyruvate derived from glucose or other sources by aerobic organisms. In this pathway, malate is produced by the action of fumarase on fumarate. In the final step of the TCA cycle, malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate using NAD^+ as the oxidizing agent. This is a highly thermodynamically unfavorable reaction ($\Delta G^0=7.1$ kcal/mole) that could lead to buildup of malate under the right circumstances. In plants capable of converting fatty acids into sugars, malic acid is formed from acetyl-CoA and glyoxylic acid under the mediation of the enzyme, malate synthase. Thus, malic acid as a starting material for the production of BUT has a major advantage over other starting materials, mainly it can be made from either petroleum products or from natural products. As a result, the production of BUT from malic acid could potentially be independent of the availability and price fluctuations of petroleum.

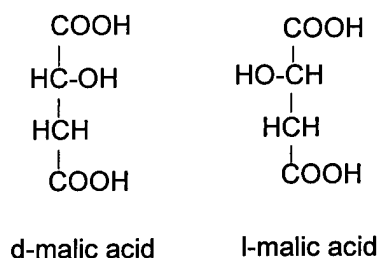
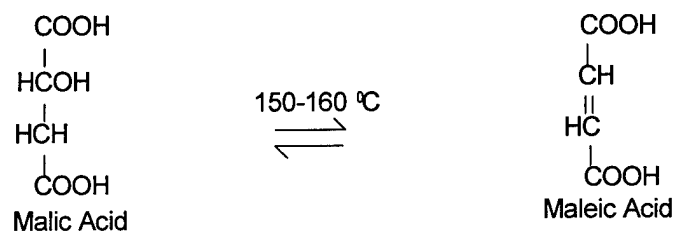


Figure 1. Structures of Malic Acid Isomers

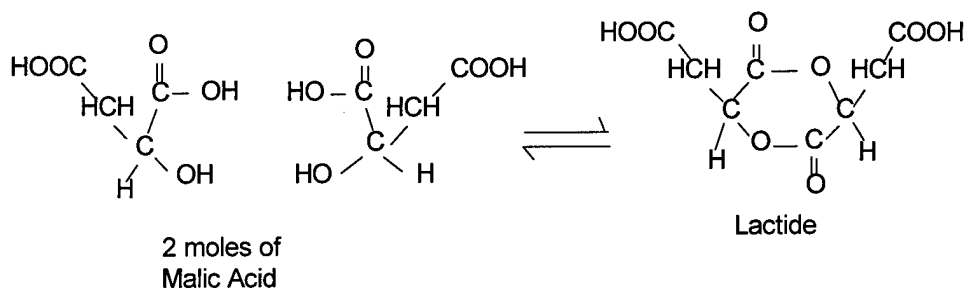
**Table I. Properties of Commercial Grade *d,l*-Malic Acid
[American International Chemical, Inc, 2000]**

Assay	99.0-100.5%
Fumaric Acid	1.0% max
Heavy Metals	10 ppm max
Maleic Acid	0.05% max
Residue on ignition	0.1% max
Water insoluble matter	0.1% max
Specific rotation (α) ²⁵ _D	Between -0.10° and +0.10°
Particle Sizes Available	Granular - 99.9% through 10 mesh sieve Fine - 99% through 25 mesh sieve Powder - 90% through 60 mesh sieve

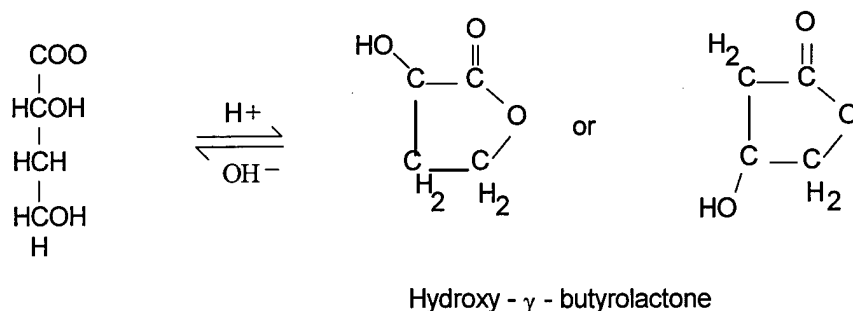
The reduction of *d,l*-malic acid to BUT has several problems which must be overcome for a successful synthesis. One of the problems is that carboxylic acids are difficult to reduce to the corresponding alcohol. The other problem is associated with the side reactions that can take place under typical chemical reaction conditions. Since malic acid is both an α -hydroxyacid and a β -hydroxyacid, it can lose water by two separate methods. As a β -hydroxyacid, malic acid is dehydrated to maleic acid when heated to temperatures between 140 and 150 °C (see Figure 2a). As an α -hydroxyacid, malic acid can self-esterify when heated to form a six-membered ring lactide (see Figure 2 b). This reaction takes place at about 80 °C. Self-esterification of malic acid to form linear polymers of various lengths can also occur. A fourth reaction can also take place as one of the carboxylic acid groups is reduced to the alcohol, *i.e.*, formation of the α or β -hydroxy- γ -butyrolactone (see Figure 2c). All of these side reactions could lead to unwanted by-products in the reduction of malic acid to BUT and/or alter the rate of the reduction reaction.



a. Dehydration of Malic Acid to Maleic Acid



b. Lactide Formation



c. Lactone Formation During Reduction of Dicarboxylic Acids

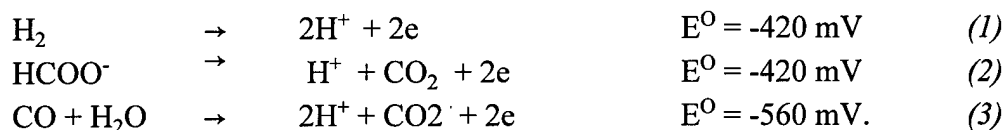
Figure 2. Reactions of Malic Acid

2.2 Microbial Reduction of Carboxylic Acids

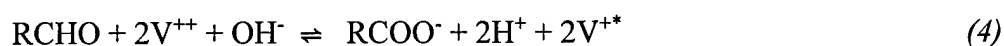
One method of catalyzing the reduction of malic acid to BUT with strict control of the by-products is through the use of enzymes and/or microorganisms in either an aqueous solution, an aqueous-organic two-phase solution, or ionic liquids. The potential for the reduction of carboxylic acids to aldehydes and subsequently to the corresponding alcohols is about -600 mV [Thauer *et al.*, 1977]. Thus, chemical reduction of carboxylic acids in aqueous solution is not possible because the protons of water are preferably reduced. Even for bacteria, this potential is difficult to overcome. Recently, there has been increased interest in microbial reduction of carboxylic acids to produce alcohols that can act as intermediates in the production of specialty compounds and pharmaceuticals. However, only a small number of anaerobic microorganisms have been found to be capable of reducing nonactivated carboxylic acids to the corresponding alcohols.

Clostridium thermoaceticum was the microbe used in this project to reduce malic acid to BUT. *Clostridium thermoaceticum* (it is more properly named *Moorella thermoacetica*) is an acetogenic anaerobic thermophilic bacterium. Acetogens have the ability to ferment a variety of sugars (including glucose, fructose and xylose) and other substrates almost exclusively to acetate. This fermentation occurs via the glycolytic pathway to pyruvate followed by further metabolism to acetate. *Clostridium thermoaceticum* also has the ability to synthesize acetate from carbon dioxide and other single carbon compounds (such as carbon monoxide and formate) via the acetyl-CoA pathway [Ljungdahl, 1992]. Because of its ability to produce acetate, *Clostridium thermoaceticum* is one of the more widely studied thermophilic bacteria.

Clostridium thermoaceticum is a strict anaerobe, *i.e.*, it is able to accept electrons supplied by H₂ (and sometimes formate, CO or the cathode of an electrochemical cell) using the following reactions:



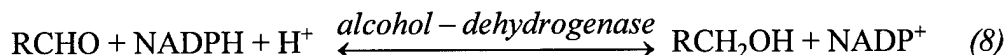
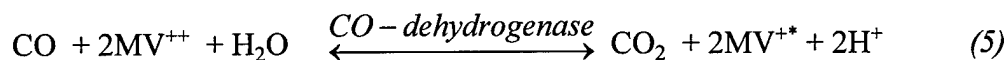
The reduction of carboxylic acids by *Clostridium thermoaceticum* is carried out by a reversible aldehyde oxidoreductase (AOR). Huber *et al.* (1995) isolated and extensively studied the AOR in *Clostridium thermoaceticum*. It exists in two forms and contains tungsten complexed to a pterin cofactor. One form is an $\alpha\beta$ dimer of apparent molecular mass of 86 kDa. The second form is described as an $\alpha_3\beta_3\gamma$ complex of 300 kDa. Both forms reversibly catalyze the dehydrogenation of aldehydes to the corresponding carboxylates at the expense of an artificial electron mediator such as viologen (V), methyl viologen (MV) or carbamoylmethyl viologen (CAV).



The $\alpha\beta$ dimer and the $\alpha_3\beta_3\gamma$ complex are sensitive to oxygen, with the reduced form more sensitive than the oxidized form.

Huber *et al.* (1995) studied the reduction of various carboxylates with the purified $\alpha\beta$ dimer AOR from *Clostridium thermoaceticum* using CAV as the electron mediator. The reactions were carried out under an atmosphere of 95% N₂ and 5% H₂ (v/v). The pH optimum for the reduction was found to be dependent on the pK_a values of the acids, shifting to lower pH with lower pK_a values. Thus, they postulated that the undissociated acid is the real substrate for the AOR and is the form that is bound to the enzyme. The reduction reaction with the purified enzyme is also temperature dependent, and this temperature dependence varies with the carboxylic acid substrate.

Further work by Huber *et al.* (1995) and Simon and Gunther (1998) utilized resting cells of *Clostridium thermoaceticum* to evaluate the ability of this microbe to reduce carboxylic acids in the presence of an artificial electron mediator. These studies were carried out under a CO atmosphere at 40 °C. The reaction solution (3 ml total) consisted of 0.4 g of packed wet cells, 33 mM (0.1 mmoles) substrate and 1 mM MV in a 0.3 M potassium phosphate buffer at pH 5.5. The presence of other enzymes in the resting cells introduces complementary reactions that allow the carboxylic acids to be reduced all the way to the corresponding alcohols:



Where MV⁺⁺ = the reduced form of methyl viologen;
 AMAPOR = artificial mediator accepting pyridine nucleotide oxidoreductase.

In contrast to the enzyme studies that only followed reaction (5) and found the optimum pH to be a function of the pK_a of the acid, the whole cell studies involved the summation of reactions (5) through (8). Reaction (6) through (8) affect the optimum pH, usually moving it to higher values than observed with the enzyme alone. The results of the resting cell studies are presented in Table II. Huber *et al.* (1995) and Simon and Gunther (1998) presented their results in terms of the Productivity Number (PN) for the sum of reactions (5) through (8). They define the PN as follows:

$$\text{PN} = \frac{\text{Amount of Product (mmol)}}{\text{Dry Weight of Catalyst (kg) X Time (hr)}}$$

If PN is high, less catalyst or time is needed to produce the product.

Table II. Reduction of Various Carboxylic Acids with Whole Resting Cells of *Clostridium thermoaceticum* [Simon and Gunther, 1998]

Substrate	PN*
Propionate	1,140
Butanoate	1,180
Succinate	1,355
γ -butyrolactone**	5
(R)-Lactate***	115
(S)-Lactate***	80

* PN calculated for one pair of electrons consumed after a reaction time of 1 hour

** after 19 hours

*** Rate is reported to increase at lower pH, but no data were given

Hideo and Wataru (1996) have also studied the ability of *Clostridium thermoaceticum* to reduce carboxylic acids. They used a two-phase system (water and a water insoluble organic solvent) with an atmosphere of either H₂ or CO. In contrast to Huber *et al.* (1995) and Simon and Gunther (1998), these researchers employed a different electron mediator, benzyl viologen, and a higher pH (pH = 5.8) for their studies. They reported up to 58% reduction of a 2% (w/v) solution of propionate under a CO atmosphere within 21 hours. This reduction appears to occur significantly faster than that reported by Simon and Gunther (1998).

Although the reduction of malic acid by *C. thermoaceticum* had not been previously studied, a related compound, succinic acid, has been reduced by resting cells of this microbe. These data indicate that the reduction of the first carboxylic group of succinic acid is rapid, however, the reduction of the second carboxylic group is slow due to the formation of the γ -butyrolactone. It appears that the reduction rate of the second carboxylic acid group depends on the rate of the hydrolysis of the lactone ring (which is slow at low pH). There was also the question of steric hindrance of the enzyme by the α -hydroxy-group, however, the ability of this microbe to reduce lactic acid seems to indicate that steric hindrance would not be an insurmountable problem.

3. Project Milestones

The milestones for the project are shown in the Gantt Chart in Figure 3 along with work completed. All the goals of the project were not met. Difficulties were experienced with the analysis methods due to the presence of high concentrations of salts in the microbial reaction medium. Due to these difficulties, Task 1 took twice as long as planned. The original microbes obtained from for this study were either not *Clostridium thermoaceticum* or they had been severely stressed. They grew, albeit very slowly. However, they failed to reduce any carboxylates. When it became obvious that the microbes were not behaving as expected, a new freeze-dried sample was ordered and received. The new sample yielded a healthy culture of *Clostridium thermoaceticum*. Both the problems with the analytical and with the original microbes served to put the project behind schedule. Because of lack of time, Tasks 4 and 6 were not completed and only preliminary work on Task 5 was accomplished.

4. Experimental Procedures and Results

4.1 Task 1 – Verify Analytical Techniques

The analytical development task set out to reliably quantify malic acid (MAL), butanetriol (BUT), and eight other compounds that might potentially be involved in the reduction of malic acid. These compounds included α -hydroxy- γ -butyrolactone (AHL), β -hydroxy- γ -butyrolactone (BHL), 2-butene-1,4-diol (BTD), maleic acid (MLE), fumaric acid (FUM), succinic acid (SUC), erythritol (ERY), and threitol (THR). For analytical purposes, this group of compounds can be divided into three categories that include the lactones, the dibasic acids, and the polyfunctional alcohols. While an HPLC method that involved direct aqueous injection and detection of these compounds would be desirable, poor detectability of the alcohols by UV absorption and the hydrophilic nature of all the analytes would make separation and detection of all these species by HPLC a slow and daunting task. The lactones, and possibly butenediol, could be directly analyzed by gas chromatography. However, derivatization of the main analytes is required for successful separation and analysis of these compounds by gas chromatography due to their polar nature and lack of volatility. The single best reagent for derivatization of both the alcohol and carboxylic acid functionalities is BSTFA (bis-trimethylsilyl trifluoroacetamide). For this reason, gas chromatography with flame ionization detection of the trimethylsilyl (TMS) derivatives was chosen as the analytical methodology.

4.1.1 Methods and Materials

4.1.1.1 Demonstrate the Separation of All Analytes as the TMS Derivatives

An initial study was conducted to demonstrate that the TMS derivatives of the analytes could be prepared and separated by gas chromatography. This study also determined the elution order and generated mass spectra of the derivatives.

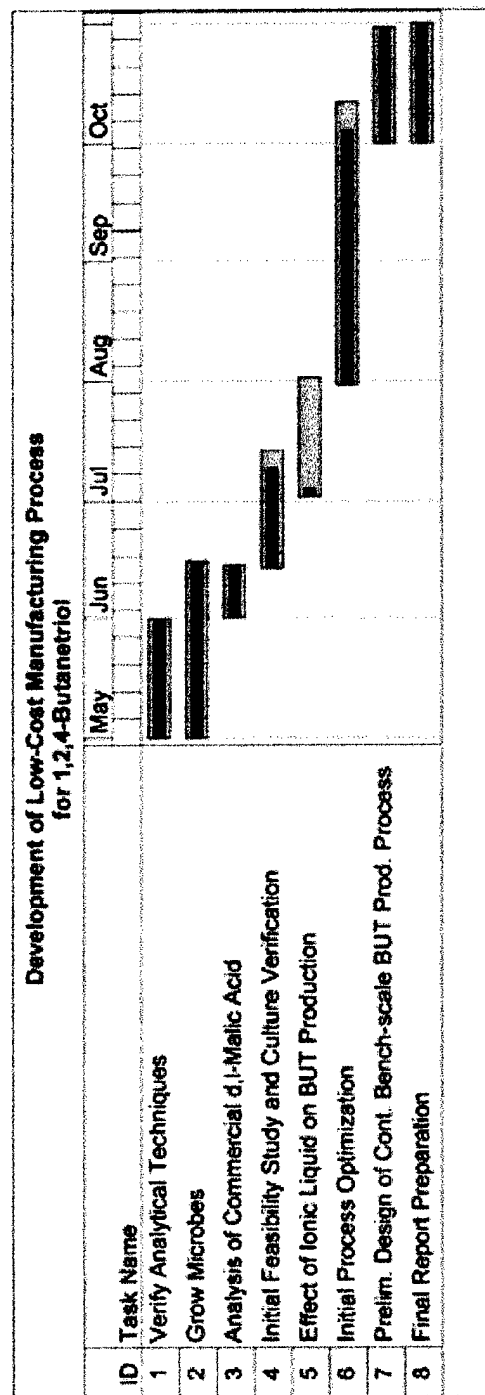


Figure 3. Time-Task Chart for the Project

Reference solutions for each of the ten analytes (See Table III for the purity of each analyte and reagent) were prepared by weighing a nominal 250 mg to the nearest 0.1 mg into 25-ml volumetric flasks. The analytes were dissolved in dimethylacetamide (DMA). DMA was chosen as a working solvent because it readily dissolved all the analytes as well as many other polar compounds and is inert to silylation reagents. To perform the analysis, 200 μ l of each individual reference solution were pipetted into individual 100-mm X 13-mm screw-capped tubes. Then, 600 μ l of pyridine and 200 μ l of BSTFA containing 1% trimethylchlorosilane were added to each tube. The tubes were shaken and 4 ml of methylene chloride were added. The tubes were placed in a 45 $^{\circ}$ C water bath for 5-10 min and subsequently allowed to stand overnight at room temperature.

The solutions were chromatographed, both individually and as a group, using a Varian 3400 GC directly coupled to a Finnigan Incos 50 mass spectrometer. Separation was accomplished using a 25-m X 0.25-mm id Supelco SPB-5 capillary column with a 0.25 μ m film thickness. Split injection mode was used with a fritted liner and an approximate split ratio of 25:1. The temperature profile was from 90 $^{\circ}$ C (3 min) to 220 $^{\circ}$ C (and hold) at 10 $^{\circ}$ C/min. The injection volume was 2 μ l.

Table III. Source and Purity of Analytes and Reagents

Analyte	Symbol	Source	Catalog #	Purity
<i>d,l</i> -malic acid	MAL	Acros	125250010	99%
1,2,4-butanetriol	BUT	Acros	107681000	95%
α -hydroxy- γ -butyrolactone	AHL	Aldrich	36,403-7	tech
β -hydroxy- γ -butyrolactone	BHL	Aldrich	42,279-7	96%
2-butene-1,4-diol	BTD	Aldrich	B8,620-6	95%
Maleic acid	MLE	Acros	125231000	99%
Fumaric acid	FUM	Aldrich	24,074-5	99+%
Succinic	SUC	Acros	2195525000	>99%
meso-erythritol	ERY	Aldrich	E260-4	Not given
<i>d,l</i> -threitol	THR	Aldrich	26,35509	97%
Dimethylacetamide	DMA	Aldrich	15,480-6	99+%
Pyridine		Acros	18022500	99+%
Methylene chloride		Aldrich	D6,510-0	99.6%
Ethyl acetate		Acros	149470010	99.5%
Trifluoroacetic acid	TFA	Acros	293810250	>99.5%
bis-trimethylsilyl trifluoro acetamide-trimethyl- chlorosilane	BSTFA	Supelco	3-3148	
Methanol		Acros	268280025	>99.8%
Acetonitrile		Fisher	A999-4	96%
Sodium chloride		Fisher	BP358-212	>99.9%

4.1.1.2 Preparation of Solutions of Analytes for Spiking and the 10DL Spiking Stock

The sample matrix used to perform the quality control tests was the reaction buffer we planned to use to conduct the carboxylic acid reduction experiments. This buffer consisted of 0.3 M phosphate buffer adjusted to pH 5.5 containing 1 mM methyl viologen (MV). The spiked buffer solutions were initially prepared using the above standards in dimethylacetamide. However, it quickly became apparent that this procedure was not satisfactory because there was too much dimethylacetamide in the sample matrix. Therefore, combination standards in aqueous media were prepared for the purpose of spiking the reaction buffer matrix. Two combination stock solutions were prepared and labeled Combo_OH(b), and Combo_AC(b). Combo_OH(b) contained ERY, THR, BUT, and BTB. Combo_AC(b) contained MAL, SUC, and FUM. Individual aqueous solutions were prepared for MLE, AHL, and BHL. The solutions were combined in a 100-ml volumetric flask and brought to volume with reaction buffer such that the final spiked concentrations represented the 10DL level (10 times the desired detection level). The concentrations of the analytes and the spiking volumes are given in Table IV. The 5DL, 2DL, 1DL, and 0.5DL concentration levels were prepared by making dilutions of the original 10DL spiked sample matrix with additional reaction buffer.

Table IV. Concentrations of Stock Solutions of Analytes and Spiking Volumes

Stock Solution	Analyte	Concentration (mg/l)	Volume of Stock to 100 ml	Concentration in 10 DL Spike (mg/l)
Combo_OH(b)	BUT	19,900	2.50	497.5
	ERY	9,732		243.3
	THR	9,612		240.3
	BTB	14,824		370.6
Combo_AC(b)	MAL	19,968	2.50	489.2
	SUC	14,340		358.5
	FUM	14,412		360.3
MLE	MLE	14,412	2.50	360.3
AHL	AHL	12,984	2.00	285.6
BHL	BHL	20,440	1.20	286.2

4.1.1.3 Preliminary Tests at the 5 and 10DL Levels

The general plan for analysis was to evaporate 4 ml of the spiked reaction buffer to dryness under a stream of nitrogen at 65 °C, reconstitute with 1 ml of DMA, add BSTFA silylation reagent and pyridine, and finally bring the volume to 4 ml with methylene chloride. Initial method testing trials were conducted by preparing 4 ml samples of the reaction buffer spiked at the 5DL and 10DL levels. These samples were then prepared for analysis by the above method.

Quantitation was accomplished using a Varian 3800 gas chromatograph with a flame ionization detector. A Gateway 600 MHz Pentium III computer with Varian Star chromatography software version 5.5 was used for data handling and instrument control. The column was an Alltech Econo-Cap EC-5 30-m X 0.25-mm id FSOT capillary with a 0.25- μ m thick coating. The phase was 5% phenyl methylsiloxane equivalent to SPB-5 (Supelco) or DB-5 (J-W). Carrier gas was helium at 18 psig inlet pressure. Split mode injection was used with a split ratio of 20:1 and an open split/splitless liner. The temperature profile for all the chromatographic runs was the same. Injection port and detector temperatures were 250 °C and 280 °C, respectively. The column oven temperature was initially 90 °C (hold for one minute) programmed at 20 °C/min to 110 °C (hold for one minute), and then programmed at 8 °C/min to 206 °C (hold for 1 minute). The injection volume was 2 μ l.

An excessive amount of time was required to blow the samples to dryness because of salt formation at the end of the process. Samples were reconstituted with 1 ml of DMA and then 225 μ l BSTFA and 375 μ l of pyridine were added. Some salts remained undissolved. The resulting solution was heated for 5 min (~50 °C) in a beaker of hot water and then 2.4 ml of methylene chloride were added to achieve a 4 ml final volume. The results from analysis of the two samples showed very poor recovery of the lactones, BTD, and maleic acid with apparent conversion of part of the maleic acid to fumaric acid.

Because of the problems associated with blowing down the samples, extraction of the analytes from the reaction buffer was attempted. Two extraction trials were conducted. Pyridine was used because the literature indicated that erythritol was slightly soluble in pyridine. In the first extraction trial, 1 gram of sodium chloride was dissolved in 4 ml of spiked reaction buffer. This solution was extracted twice with 3 ml portions of 10% methanol in pyridine. The second extraction resulted in a large portion of the aqueous phase being absorbed into the organic layer. The combined extracts were blown to dryness, derivatized, and analyzed as described above. In the second extraction trial, 1 gram of sodium chloride was dissolved in 4 ml of the spiked reaction buffer as above, then extracted twice with 3 ml portions of 10% pyridine in ethyl acetate. The combined extracts were then blown to dryness, and derivatized before analysis.

The analyses of these extraction samples showed that the recoveries of the dibasic acids and lactones were marginally adequate. Recovery of BUT was poor and recovery of MAL, ERY, and THR was nearly zero.

Extra peaks (not in the blanks) were observed indicating that the silylation reaction may not be going to completion. Therefore, a quick study of the silylation method was conducted. Aliquots of the BUT, MAL, and ERY in DMA were placed in screw-capped vials and treated with either 50 or 100 μ l each of BSTFA and pyridine. They were heated for 5 minutes at 50 °C and diluted to 4 ml with methylene chloride. Even the samples with the 100 μ l addition of BSTFA showed incomplete derivatization of the malic acid and erythritol. This study was repeated using 150 μ l of

BSTFA and pyridine. Again the malic acid was not completely derivatized. Since this represented a several fold excess of reagent, it was postulated that the samples required longer heating times. The experiment was then repeated and the vials heated in a water bath at 50 °C for one hour. Complete derivatization was observed.

The method plan changed at this point to effect a separation of the salts from the analytes. As a preliminary to this procedure, a solubility test was performed. Fifty mg of ERY were placed in each of two tubes. Four ml of acetonitrile were added to one tube and 4 ml of methanol to the other. After shaking the MeOH dissolved the ERY, but even after warming to 50 °C the acetonitrile failed to dissolve the ERY. However, after the addition of 0.8 ml of water to the acetonitrile, dissolution of the ERY was accomplished. Two variations on an evaporate and extract procedure were then tried. Two 4-ml samples of the 10DL spiked reaction buffer were blown to near dryness. One sample was extracted twice with 3 ml portions of methanol and the other sample was extracted twice with 3 ml portions of 85%acetonitrile/15%water. The combined extracts of each sample were then evaporated to dryness and reconstituted with 800 μ l of DMA and 400 μ l each of BSTFA and pyridine. The derivatization mixtures were then heated on a water bath at 50 °C for 1 hr before dilution back to 4 ml with methylene chloride. GC-FID analysis of the methanol extract showed no recovery of the MAL, MLE and very poor recovery of AHL, BHL, and BTM. The acetonitrile extract was better but still had low recoveries of AHL, BHL, and BTM.

Four variations on the above procedure were tried. In all of these variations, the spiked reaction buffer sample was extracted twice with 3 ml of 85% acetonitrile/15% water and then various additions of DMA and trifluoroacetic acid were used. Each of the four 4-ml 10DL spike samples received one of the following additions: (1) 200 μ l DMA, (2) 25 μ l neat trifluoroacetic acid, (3) 200 μ l 1:10 aqueous trifluoroacetic acid, and (4) 200 μ l of DMA and 1:10 aqueous trifluoroacetic acid. The samples were evaporated down to a 400-500 μ l volume as indicated by the round part of the bottom of the 13-mm X 100-mm test tube. These samples were reconstituted with 1 ml of DMA and then 400 μ l each of pyridine and BSTFA. They were then heated on a water bath for one hour at 50 °C and analyzed by GC-FID. The best recoveries were obtained with the combined 1:10 trifluoroacetic acid and DMA addition, but the MLE recovery was still only around 50%.

4.1.1.4 Final Analytical Method

The final procedure as given below substituted 100% acetonitrile for the first extraction, used 100 μ l of 1:10 trifluoroacetic acid and added the acid only after the first aqueous evaporation.

A 6-ml mixture containing the microbial cells, viologen and the reaction buffer slurry was placed in a 13-mm X-100 mm screw-capped test tube. The sample was then centrifuged for 30 min at 3000 rpm and 10 °C in a Beckman GPR centrifuge with a swinging bucket rotor. After separation of the cells, 4 ml of the supernatant was drawn off and transferred to a separate test tube. Then, 200 μ l of DMA

were added to the supernatant. This solution was subsequently blown down to approximately 1 ml (est.) in a N-Evap Organomation Model 112 under a stream of nitrogen with a water bath temperature of 50 °C. Following the addition of 1 ml of acetonitrile, the contents of the test tube were briefly shaken causing most of the salts to precipitate. The tube was then returned to the N-Evap and the contents blown down to approximately 400 μ l (est.). Three ml of acetonitrile and 100 μ l of a 1:10 aqueous trifluoroacetic acid were then added to the test tube. The contents of the test tube were vigorously shaken and the tube placed in an ultrasonic bath for 5 min at 50 °C. The mixture was then centrifuged at 2700 rpm for 15 min at 10 °C. The supernatant was drawn off with a Pasteur pipette and transferred to a separate tube. The precipitated pellet was washed with an additional 3 ml of 85% acetonitrile/15% water. The contents of the tube were vigorously shaken, heated for 5 min in a sonic bath and centrifuged at the same conditions as previously stated. The resulting supernatant was drawn off and combined with the first supernatant. The combined solution was then blown to nearly dryness (less than 50 μ l) in a N-Evap at 50 °C. At this point, the sample was usually stored overnight in the refrigerator. The sample was reconstituted by adding 1 ml of DMA followed by 400 μ l of pyridine. Trimethylsilyl derivatization was accomplished by adding 400 μ l of BSTFA containing 1% trimethylchlorosilane. The mixture was shaken and the sealed tube was placed in a 50 °C water bath for one hour. The cooled sample was brought to 4 ml final volume with the addition of 2.2 ml methylene chloride. Quantitation was accomplished using capillary gas chromatography with a flame ionization detector as described above. Approximately, three working days were required to complete the analysis of samples using this methodology.

4.1.1.5 QC Trials and Detection Limit Determination

This final analytical method was subjected to quality control trials. To accomplish these trials, samples of the reaction buffer were spike at the 10DL, 5DL, 2DL, 1DL and 0.5DL levels, where DL is the desired detection limit (See Table IV for the concentration of the 10DL spike). The samples were analyzed on four consecutive days to determine the precision and accuracy of the method and the detection limit.

4.1.2 Results of Analytical Verification Task

4.1.2.1 GS- MS Separation of TMS Derivatives of Analytes

The resulting chromatogram of the GC-MS analysis of the TMS derivatives of the analytes is shown in Figure 4. As can be observed from the figure, separation of all the analytes occurred under the conditions of this analysis. The mass spectra of the TMS derivatives of the individual analytes are compiled in Appendix A.

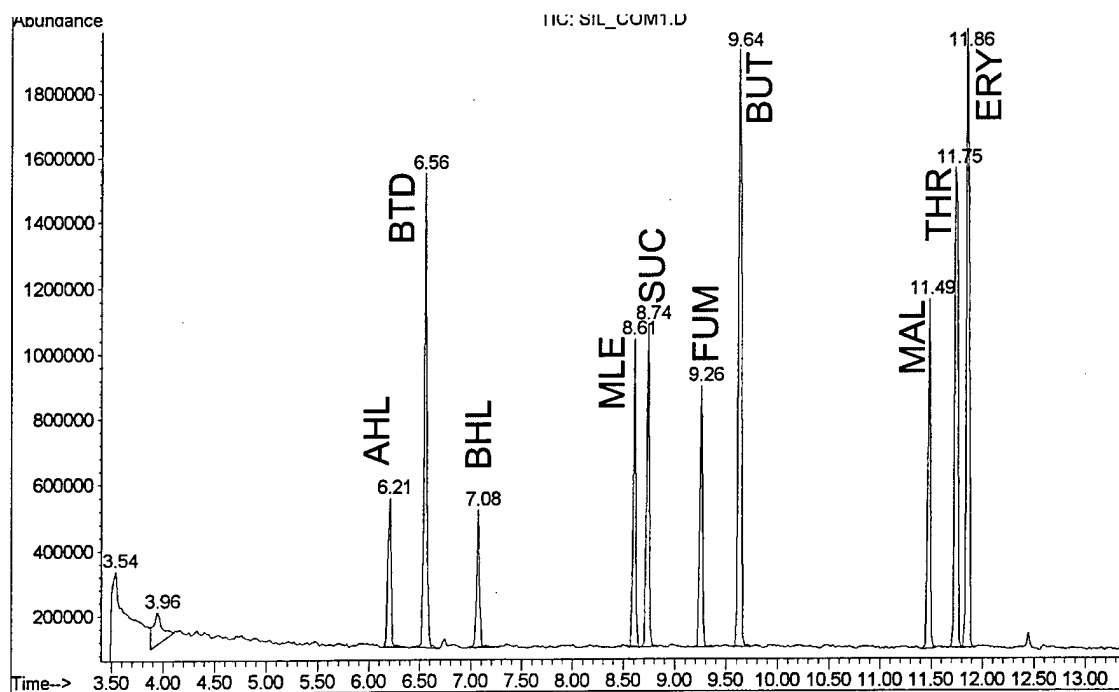


Figure 4. GC-MS of Analyte Mixture

4.1.2.2 Results of QC Trials

The detection limits calculated by U.S. Environmental Protection Agency method detection limit procedure (USGC, 1999) are summarized in Table V. The data from the QC trials are compiled in Appendix B. The data show that the method was highly variable, the variability being due to the high salt concentration in the reaction buffer. The detection limits were higher than desired and the recoveries were generally low. To help overcome these problems with the analytical methodology, the amount of salts in the reaction buffer was cut from 0.3 M (the concentration recommended by Simon and Gunther (1998)) to 0.1 M (the concentration recommended by Hideo and Wataru (1995)). Due to time constraints of the program, the QC data were not rerun.

Table V. Detection Limits for Analytes in 0.3 M Reaction Buffer

Compound	Detection Limit (mg/l)
BUT	221.4
ERY	108.6
THR	107.4
BTD	131.8
MAL	186.5
SUC	160.5
FUM	120.9
MLE	109.2
AHL	140.2
BHL	100.2

4.2 Task 2 – Grow Microbes

4.2.1 Methods and Materials

4.2.1.1 Source of Microorganisms

The microorganisms used in this study were purchased from the American Type Culture Collection (ATCC) located in Manassas, Virginia. Two different strains of *Clostridium thermoaceticum* were obtained for use in this project, 35608 (DSM 521) and 39073. These strains were provided by ATCC in freeze-dried form in sealed ampoules under nitrogen atmosphere. The original strains were purchased during the month of June 2001. The package was shipped on a Thursday and did not arrive in our facility until the following Monday. The ampoule of 39073 was broken upon arrival and replaced by ATCC from the same batch. The efficacy of both these microbes later came in question (See Sections 4.2.2 and 4.4.2). They were replaced in August 2001 with new freeze-dried cultures from a different batch. However, due to time constraints, only 35608 was grown and utilized in further research work.

4.2.1.2 Experimental Setup for Growth of *Clostridium thermoaceticum*

Liquid cultures of *Clostridium thermoaceticum* were grown in 500-ml Erlenmeyer flasks. Each flask was fitted with a bubbler consisting of a butyl rubber stopper through which two 6-mm glass tubes were passed. The inlet tube was long enough to reach the bottom of the flask. A small piece of cotton was inserted into the top of this tube to filter out any microbes in the incoming gas. Rubber septa (7-mm, Aldrich Z12434-6) were inserted into the top of both tubes (See Figure 5). The bubblers were sterilized separately from the flasks.

The setup used to provide humidified carbon dioxide to each flask is shown in Figure 6. To attach a flask to the carbon dioxide gas, an one-inch, 20-gauge stainless steel needle (Aldrich Z19,251-1 with the plastic hub removed) was inserted through the septum on the inlet tube of each bubbler. The other end of the needle was inserted into 1/32-inch id C-flex tubing (Aldrich T8413) that was in turn connected to a gas manifold via a second stainless steel needle inserted into the other end of the tubing. C-flex tubing was chosen to minimize oxygen passage into the system. Carbon dioxide gas (Coleman grade from Air Products, Inc.) was first passed through a bubbler containing sterile deionized water to humidify the gas stream. Polyethylene tubing was used for the connections between the tank regulator, the bubbler, and the manifold.



Figure 5. Photograph of Growth Flask and Bubbler

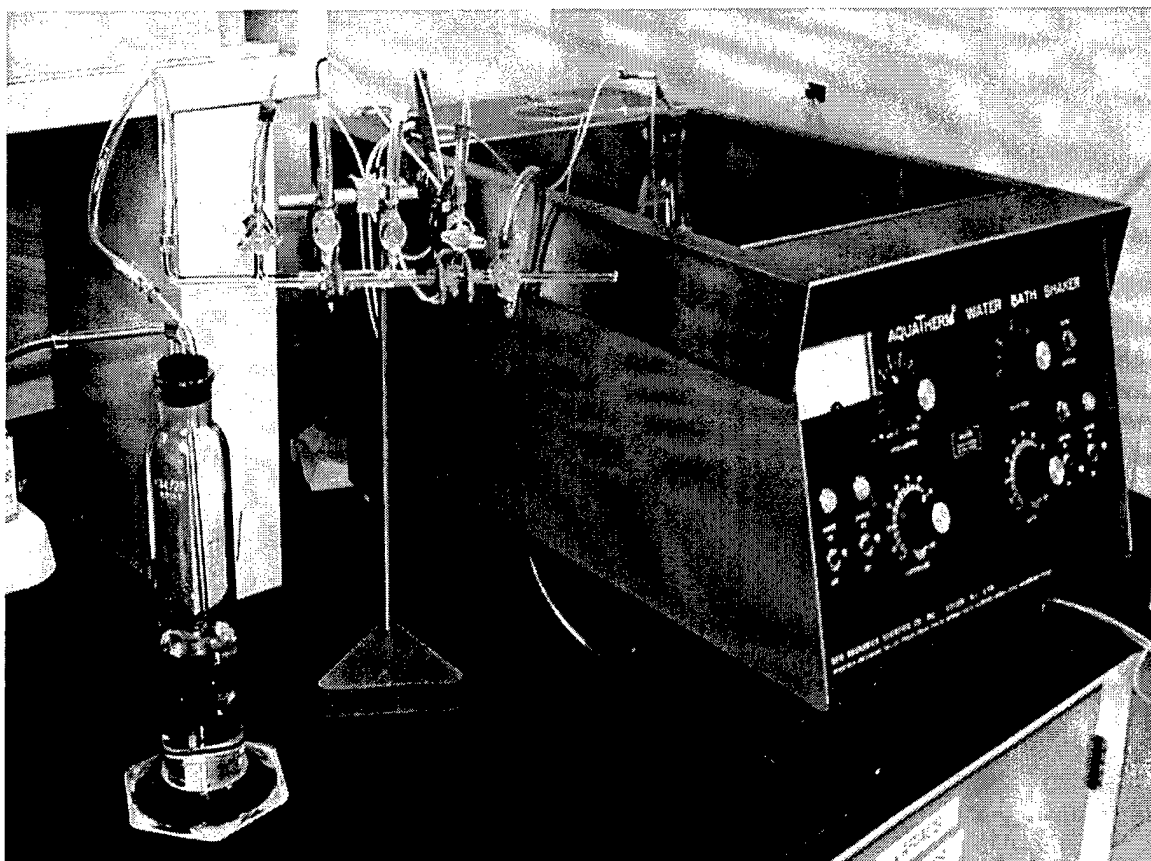


Figure 6. Photograph Showing Bubbler, Gas Manifold and Water Bath Shaker

To prevent the back flow of air into the flasks, the gas exiting the flask was run through a condensation trap and a bubbler. An one-inch, 20-gauge stainless steel needle was inserted into the septum of the exit tube of each bubbler. The other end of the needle was inserted into silicon tubing. A second needle was attached to the other end of this tubing. This needle was inserted in a septum that covered a receiving flask adapter. This adapter was used to collect condensation from the growth flasks. The exit of the adapter was connected to a bubbler via polyethylene tubing. The exit gas from the bubbler was exhausted into a chemical hood with a face velocity of 100 linear feet per minute.

The media used to grow both 35608 and 39703 was a modification of that described by Ljungdahl and Andreesen (1978). The constituents of the media are shown in Table VI. The buffer solution, 'C', was prepared first and divided equally (90 ml) into four 500-ml Erlenmeyer flasks. The flasks were plugged with foam stoppers and autoclaved at 250 °C and 16 psig for 30 minutes in a Barnstead Laboratory Sterilizer Model C2250. After removal of the flasks from the autoclave, each flask was fitted with a sterile bubbler. The flasks containing the buffer solution were placed in a New Brunswick Scientific Co. Aqua Therm Water Bath Shaker maintained at a temperature of 58 °C. The inlet and exit gas lines were inserted into the bubblers and the carbon dioxide gas flow was adjusted to obtain even bubbling in all flasks. The carbon dioxide was

Table VI. Growth Medium for *Clostridium thermoaceticum* [Simon and Gunther, 1998; Ljungdahl and Andreessen, 1978; Hideo and Wataru, 1995]

Soln.	Ingredient	Conc. of Soln. Added	Source	Catalog #	Purity	Amount	Final Conc. (mM)
A	Glucose (Dextrose)		Fisher	BP350-1	MB Grade	21.6 g	100
	Deionized Water					180 ml	
	Bacto Yeast Extract		Difco	212750		6.0 g	
B	Tryptone Peptone		Difco	211705		6.0 g	
	$(NH_4)_2SO_4$	12.0 g/100 ml	Acros	42340-5000	ACS reagent	10.0 ml	7.6
	$MgSO_4 \cdot 7H_2O$	10.0 g/100 ml	Aldrich	24,697-2	99%	3.0 ml	1
	$Co(NO_3)_2 \cdot 6H_2O$	2.0 g/100 ml	Fisher	S79973	98.0-102.0%	1.8 ml	0.1
	$Na_2WO_4 \cdot 2H_2O$	0.398 g/100 ml	Acros	42447-0050	ACS Reagent	1.0 ml	0.01
	$Na_2MoO_4 \cdot 2H_2O$	0.288 g/100 ml	Aldrich	22,105-8	99+%	1.0 ml	0.01
	Na_2SeO_3	0.040 g/100 ml	Acros	20073-0250	44-46% Se	0.6 ml	0.001
	$NiCl_2 \cdot 6H_2O$.0268 g/100 ml	Acros	20767-0250	97%	0.6 ml	0.001
	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$		Aldrich	20,350-5	99.997%	0.0468 g	0.1
	$Na_2S_2O_4$		Fisher	S310-100	Purified	0.209 g	0.1
	Sodium thioglycolate		Acros	148890-500	98%	0.60 g	4.4
	Deionized Water					642 ml	
C	$NaHCO_3$		Acros	42427-0010	ACS Reagent	12.0 g	200
	K_2HPO_4		Fisher	BP363-500	>99.0%	8.4 g	40
	KH_2PO_4		Fisher	BP362-500	>99.0%	6.6 g	40
	Deionized Water					360 ml	

allowed to bubble through the flasks for at least one hour. This bubbling served to degas the solution and lower the pH of the buffer solution to less than 8.4.

During the time required to degas and lower the pH of the buffer solution, solutions 'A' and 'B' were prepared and sterilized. Solution 'A' was prepared by weighing the glucose into a 500-ml Erlenmeyer flask and adding 180 ml of deionized water. The flask was plugged with a foam plug. Solution 'B' was prepared by weighing the tryptone peptone and yeast extract into a 1-liter Erlenmeyer flask. Deionized water (500 ml) was added to the flask and the contents swirled to dissolve the powders. Stock solutions of all the stable salts for the 'B' solution were made in deionized water. The appropriate amounts of these stock solutions were pipetted into the flask containing the tryptone peptone and the yeast extract. The ferrous ammonium sulfate, dithionite (sodium hydrosulfite), and sodium thioglycolate were weighed and added as solids along with an additional 142 ml of deionized water just prior to autoclaving. The initial media used to grow the microbes did not contain sodium thioglycolate as recommended by Simon and Gunther (1998). Later, sodium thioglycolate was added to the growth media, as recommended by Hideo and Wataru (1995). The microbes grew better on the media containing the sodium thioglycolate. The flask was plugged with a foam plug. Solutions 'A' and 'B' were then autoclaved at 250 °C and 16 psig for 30 minutes. As soon as the autoclave cooled enough to open the door, the 'A' and 'B' solutions were removed from the autoclave. Forty-five ml of solution 'A' and 160.5 ml of solution 'B' were then immediately added to each flask containing solution 'C'. The carbon dioxide was allowed to flow through the media for at least an additional hour to remove all the oxygen from the system.

The prepared media was inoculated with the microbe. Initially the freeze-dried culture was added directly to the prepared media. Thereafter, 12 ml of a previously grow culture were aseptically transferred to each flask. The water bath shaker was then covered to maintain the temperature at 58 °C and the shaking speed set at 125 rpm. The cultures were allowed to grow until they entered the resting phase as determined by the optical density of the media at 540 or 580 nm. The optical density was determined with an Aurora Instruments Spectrophotometer using matched 10-mm quartz cells purchased from Starna Cells, Inc. When the cultures reached the resting phase, the bubbler was removed from each flask, and the flasks were immediately sealed with butyl rubber stoppers. If the cells were not to be harvested immediately, the stoppered flasks were stored in the refrigerator at 7 °C.

4.2.1.3 Harvesting of the Microbial Cells

All handling of the liquid cultures and the harvested microbial cells was performed in a nitrogen atmosphere in a Labconco controlled atmosphere glove box. To harvest the cells from the liquid culture, the liquid from each flask was poured into a 500-ml Corning polypropylene conical bottom centrifuge bottle (Fisher 07-200-621) and the bottles tightly sealed. The bottles were centrifuged in an IEC Centra-8R Centrifuge at 2000 rpm and 24 °C for 40 minutes. After centrifugation, the bottles were placed back in the glove box. The liquid was decanted, and the cells were shaken with 100 ml of 0.01 M

phosphate buffer, pH 7.0. The centrifugation step was repeated. The liquid was again decanted. Then, 50 ml of phosphate buffer (0.01 M, pH 7.0) were added to the bottle and the cells resuspended. Ten ml of suspended cells were pipetted into each of five 15-ml Falcon polypropylene conical bottom centrifuge tubes (Fisher 14-959-70C). The tubes were tightly sealed and then they were centrifuged at 2000 rpm at 24 °C for 40 minutes. After centrifugation, the liquid was discarded and 10 ml of phosphate buffer were added to each tube. The cells were resuspended by mixing with an Abbott Laboratories Vortexer. The tubes were recentrifuged and the supernatant discarded. These triply washed cells were used for all reduction experiments in Tasks 4 and 6. If the cells were to be used within 48 hours of harvesting, they were placed in the glove box in sealed tubes. If the cells were to be held longer before use, they were frozen at -18°C in a non-frostfree freezer (Hotpoint).

4.2.1.4 Determining the Growth Curves of the Microbes

The growth curves of the microbes were determined using the same setup as described for the growth of the microbes in Section 4.2.1.2 except that the exit tube of the bubbler was replaced with a rubber septum. A 6-inch, 18-gauge needle with a Leuer lock (Aldrich Z26,135-1) was inserted through the septum. This needle served as the exit for the carbon dioxide gas flowing through the media. Growth studies were conducted at 58 °C and 65 °C (the growth temperature suggested by Simon and Gunther (1998)).

When a sample of the media was to be taken (approximately every 2 hours), the cover was removed from the bath and the shaker stopped. The flow of carbon dioxide into the flasks was maintained. A ten-ml syringe (with the plunger pushed to zero) was attached to the Leuer of the needle in the flask to be sampled. The flask was swirled to thoroughly mix the media and cells. The needle was then pushed down into the middle of the solution and 3 ml of liquid drawn into the syringe. The needle was pulled up and out of the solution, and the syringe containing the sample carefully removed. Care was taken during this operation to avoid introducing any air into the flask. The sample was immediately placed in a 10-mm quartz cell. The optical densities of the samples were measured at 540 nm or 580 nm with an Aurora Instruments Spectrophotometer.

The wavelengths utilized for the optical density readings were chosen after the absorbance of the media (no microorganisms) as a function of wavelength was determined. The absorbance of the media without sodium thioglycolate showed a minimum in the 540 to 660 nm region. The media containing the sodium thioglycolate had some absorbance at 540 nm, therefore, the wavelength used for the optical density readings was shifted to 580 nm.

4.2.2 Growth Study Results

The absorbance readings of the cultures were corrected for the absorbance of the quartz cell and the media at the wavelength of measurement. These results were

plotted as a function of time. The results for the original 39073 and 35608 cultures are shown in Figures 7. These plots indicate an exceptionally long lag period before the microbes entered the logarithmic growth phase. These microbes appeared to be highly stressed. The cultures had a dull appearance and were clumpy. Both of these microbes failed to grow at 65 °C. Experiments with these microbes in Task 4 showed no reduction succinic or malic acids and only minimal reduction of butyric acid.

A new culture of 35608 was ordered from ATCC in August 2001. This culture was shipped overnight and the packaging received in good shape. This culture was started from the freeze-dried sample and transferred three times before the growth study was repeated. In contrast to the previously obtained culture of 35608, this new microbe yielded a healthy, rapidly growing culture. In contrast to the previous culture, this new culture had a creamy, pearlized appearance (See Figure 8).



Figure 8. Photograph of New Culture of 35608

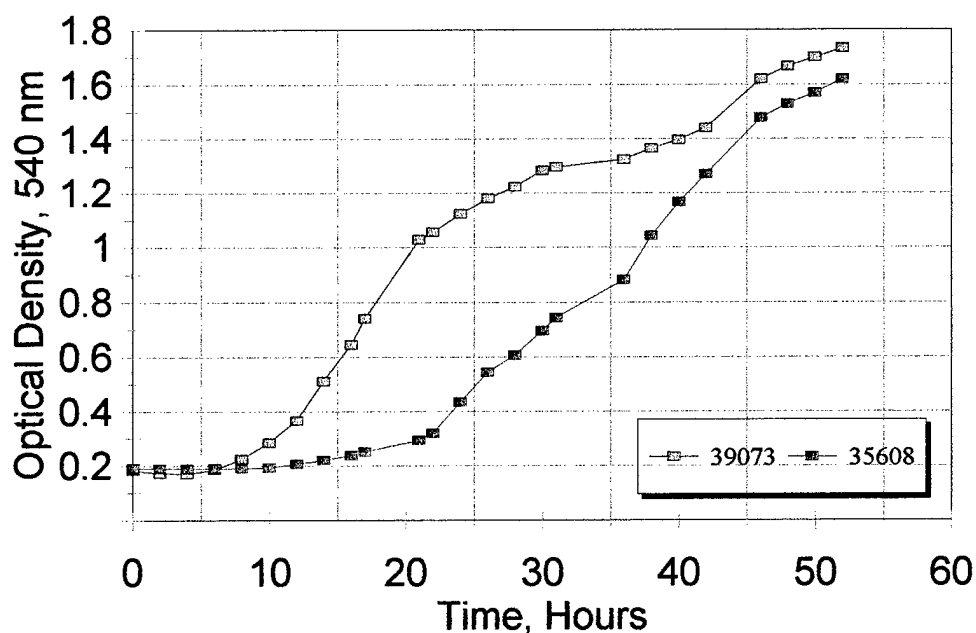


Figure 7. Growth Curves for the Original Cultures of 39073 and 35608

A repeat of the growth study with this new culture is shown in Figure 9. The growth curve of the new 35608 microbe is more normal than that obtained for the old culture. This curve shows that 35608 enters the resting phase in a little over 20 hours compared to the 48 hours required for the old culture.

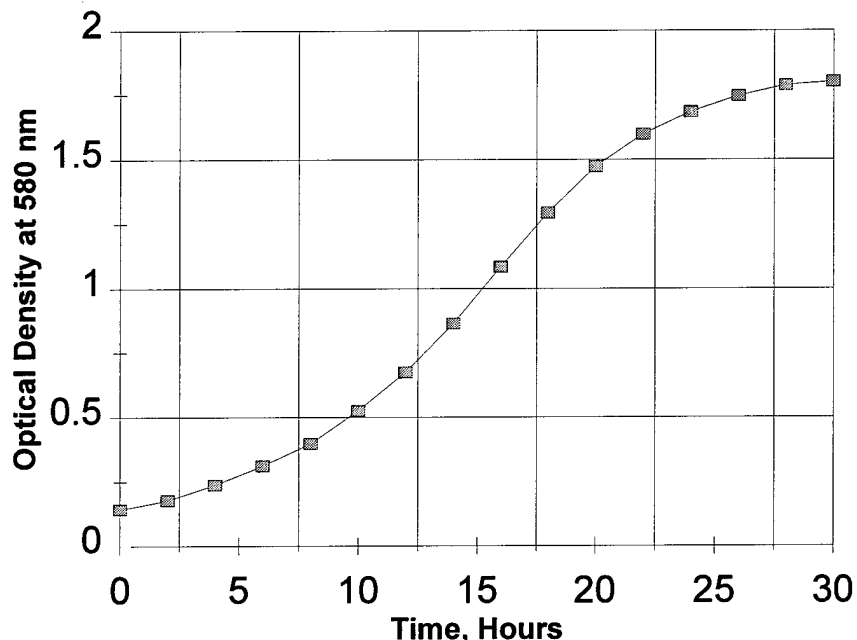


Figure 9. Growth Curve for New 35608 Culture

4.3 Task 3 - Analysis of Commercial Grade Malic Acid

4.3.1 Methods and Materials

Commercial grade malic acid was obtained in a 50 lb cardboard barrel from Penta Manufacturing Co. To obtain a representative sample of this material for analysis, the contents of the drum were mixed and several random samples were taken. The samples were then combined and mixed. Three nominal 10 gram subsamples of this sample were randomly taken for analysis. One gram of each subsample was weighed into a 25-ml volumetric flask and then DMA was added to bring the volume to 25 ml. One ml of this solution was treated with 400 μ l each of BSTFA and pyridine and the mixture heated on a water bath at 50 °C for one hour. Then, methylene chloride was added to bring the volume to 4 ml. The resulting solution was chromatographed using the conditions describe in Section 4.1.1.3.

4.3.2 Results of Commercial Grade Malic Acid Analysis

The analysis of the three samples of commercial grade malic acid showed that the purity of this malic acid ranged from 99.34 to 99.47%. The major impurity was

fumaric acid (See Appendix C) with concentrations ranging from .53 to .66%. Succinic and maleic acids were also present, however, their concentrations were too low to quantify. The presence of fumaric acid in the malic acid from the manufacturing process is expected. The commercial material that we purchased was within the specifications given by the manufacturer.

4.4 Tasks 4 and 6 - Initial Feasibility Study and Culture Verification and Initial Process Optimization

Our initial approach was to utilize the generalized experimental conditions reported by Simon and Gunther (1998) to demonstrate the feasibility of microbially reducing *d,l*-malic acid to 1,2,4-butanediol with *Clostridium thermoaceticum*. We were then to determine if the reaction would take place in the presence of an ionic liquid and later optimize the process in Task 6. To determine if the ATCC 35608 *Clostridium thermoaceticum* (the same strain as that used by Simon and Gunther, 1998) was actively reducing carboxylate groups, we planned to also run the reaction succinic acid and then compare our data on succinic acid with the published data of Simon and Gunther (1998). Unfortunately, problems with the original microbes received from ATCC forced us to revise our approach and essentially Tasks 4 and 6 were combined into one task. Because of the time required to analyze malic and succinic acids and their corresponding alcohols, we also later revised our approach to use butyric acid to show that the new microbes were reducing the carboxylic acid group. Butyric acid was chosen because it could be analyzed by direct aqueous injection on the GC. This cut the analysis time from 3 days to less than one day.

4.4.1 Methods and Materials

4.4.1.1 Preparation of solutions

The chemicals utilized in the preparation of the carboxylate solutions for reduction with the microbes are listed in Table VII.

Table VII. Chemicals Used in Tasks 4 and 6 and Their Purities

Chemical	Source	Catalog #	Purity
KH_2PO_4	Fisher	BP362-500	>99.0%
Malic Acid	Penta Manf.		>99.3%
Sodium malate	Aldrich	30,849-8	97%
Potassium hydroxide	Fisher	P250500	86.9%
Phosphoric acid	Aldrich	21,510-4	85%
Butyric acid	Acros	108110050	99+%
Succinic acid	Acros	21955250	>99%
Sodium succinate	Aldrich	22,473-1	99%
Argon gas	Air Products		Ultra pure carrier grade
Carbon monoxide gas	Air Products		99.3%

All phosphate buffers used in this study were prepared by dissolving the appropriate amount of monobasic potassium phosphate (KH_2PO_4) in deionized water. The resultant solution was titrated to the desired pH with 2 N potassium hydroxide. The pH was monitored with an Orion Model 420A pH/mV/ $^{\circ}\text{C}$ meter with an Orion triode electrode. The solution was then autoclaved at 250 $^{\circ}\text{C}$, 16 psig for 30 minutes and transferred into a sterile 125-ml Wheaton bottle (Aldrich Z11,401-4). The bottle was then sealed with a butyl rubber septum (Aldrich Z16,606-5) and an aluminum crimp seal (Aldrich Z11,414-6). A 4-inch, 21-gauge needle connected via C-Flex tubing to a gas manifold was inserted into the septum. A second 1½-inch, 21-gauge needle was inserted into the septum to act as the exit for the gas. Removal of oxygen from the solution was accomplished by bubbling argon gas through the solution for 1 hour. After 1 hour, the exit needle was removed and the bottle pressurized with argon before the inlet needle was removed. The deaerated solution was stored in the glove box under a nitrogen atmosphere.

Solutions of the carboxylates were made by dissolving a weighed amount of the carboxylic acid or its sodium salt in the appropriate buffer and titrating with potassium hydroxide or phosphoric acid to the desired pH. The solutions were sterile filtered through a 0.2- μ Nalgene disposable filter. Sterile filtration was used to prevent any unwanted esterification reactions that might occur during autoclaving. This sterile filtration also helped to deaerate the solution. The sterilized solution was placed in a 30-ml sterile Weaton vial (Aldrich, Z11,398-0). The vial was sealed with a septum and aluminum crimp seal. Further deaeration was accomplished by bubbling argon gas through the solution for 30 minutes. The carboxylate solutions were stored in the glove box under a nitrogen atmosphere.

The viologens were prepared as 0.03 M solutions in deionized water. After sterile filtration, the solutions were placed in sterile 30-ml Weaton vials and sealed. They were deaerated by bubbling argon gas through the solutions for 30 minutes. The bottles were then stored in the glove box under a nitrogen atmosphere.

4.4.1.2 Initial Experimental Setup for Carboxylate Reduction

The initial concept was to use a gas sparging technique to provide carbon monoxide to the microbes for carboxylate reduction. The gas sparging also was to provide mixing of the solution. This work was conducted in 30-ml Weaton vials (Aldrich Z11,398-0) with butyl rubber septum and aluminum crimp seals. As shown in Figure 10, a 6-inch, 20-gauge, stainless steel needle attached to C-flex tubing was used as the inlet gas bubbler. The outlet for the gas was a 1-inch, 20-gauge needle. This needle was attached to C-flex tubing that was vented in the back of the hood.

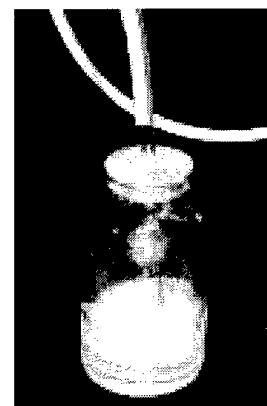


Figure 10.
Photograph of
Reaction Vial

For the initial proof-of-concept experiments, 1.6 ml (~1.6 g) of washed microbes (old 35608 and 39073), the appropriate amount of carboxylate solution (either commercial grade malic acid, sodium malate, butyric acid, or sodium succinate adjusted to the desired pH), and 1 mM of an electron mediator (methyl viologen or benzyl viologen) were added to the vials. Sufficient phosphate buffer (0.1 or 0.3 M adjusted to the desired pH) was then added to make a total volume of 6 ml. The addition of the various components to the vials was accomplished under a nitrogen atmosphere in a glove box. Once the materials were added to the vials, the butyl rubber septum and crimp seals were placed on the vials. The vials were placed in a Quincy Labs Model 10-140 incubator located in a chemical hood. The incubator temperature was maintained at 40 °C. The inlet and outlet gas lines were then connected to the vials. These gas lines

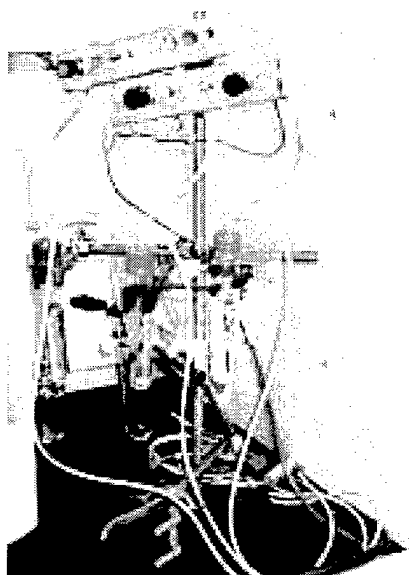


Figure 11. Photograph of the Inlet Gas Manifold

were run out of the top of the incubator (through the thermometer hole) and either connected to the inlet gas manifold or vented into the back of the hood. Initially, the carbon monoxide gas from the tank was directed into a septum manifold and then into the individual vials. Foaming of the samples and loss of the sample via the exit gas stream was a major problem in the initial trials. To provide greater control over the gas flow, this septum manifold was replaced with a manifold that consisted of three gas chromatographic grade regulating valves (Porter Instruments). These valves deliver a constant gas flow against a variable back pressure (See Figure 11). The gas flow was adjusted to minimize foaming, but still provide sufficient carbon monoxide to accomplish the reduction of the viologens as indicated by the blue or purple color of the solution.

4.4.1.3 Revision # 1 of the Experimental Setup

The reactor design was revised to minimize the sample loss encountered with the 30-ml vials. The reaction vessel chosen was a 25-ml pear-shaped flask with a 14/20 ground glass joint and a side arm (Kontes, 294500-0025). The ground glass joint was fitted with a 100 ml ball tube (Aldrich Z10,059-5) with 14/20 ground glass joints. A small amount of glass wool was placed in the ball tube to help break up any foam. Rubber septa (Aldrich Z10,076-5 and Z10,074-9) were placed in the top outlet of the ball tube and the side arm of the pear flask (See Figure 12). The inlet gas needle was inserted into the septum in the side arm of the flask. The outlet needle was inserted into the septum in the top of the ball

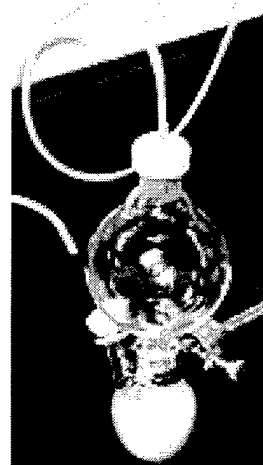


Figure 12. Photograph of Pear-shaped Reactor and Ball Tube

tube. The whole apparatus was placed in the incubator located in the hood. The inlet and outlet gas lines were run through the thermometer port of the incubator and connected to either the inlet gas manifold or the exhaust. Samples for reduction were prepared as previously described except that all amounts were doubled to give a final volume of 12 ml.

Again, the carbon monoxide gas flow was adjusted to minimize foaming while still providing sufficient gas for the reaction to occur. This setup was marginally acceptable. The foam caused a significant portion of the microbes to be carried out of the pear-shaped flask into the ball tube where they dried out. Some sample loss also occurred.

4.4.1.4 Revision # 2 of the Experimental Setup

The flow-through gas system was abandoned in favor of a static system with a large gas to liquid ratio. For this system, 125-ml storage flasks for air-sensitive materials (Aldrich, Z10,328-4) were employed. These flasks, shown in Figure 13, are sealed with a Teflon stopcock septum outlet. To prepare the flasks, a septum (Aldrich, Z-12436-2) was placed on the outlet tube of the stopcock and a vacuum hose with a stopcock was attached to the 'T' outlet. A 6-inch stainless-steel, 20-gauge needle was inserted through the septum and the stopcock into the flask. The other end of the needle was attached to the gas manifold via C-flex tubing. The gas manifold arrangement was altered so that either argon or carbon monoxide gas could be flowed into the flask. The flasks were purged for 15 minutes with argon gas and then the exit stopcock was closed. The needle was pulled out of the flask (but not through the septum) and the inlet stopcock was closed. The needle was removed from the septum and the flask placed in the glove box under an atmosphere of nitrogen.

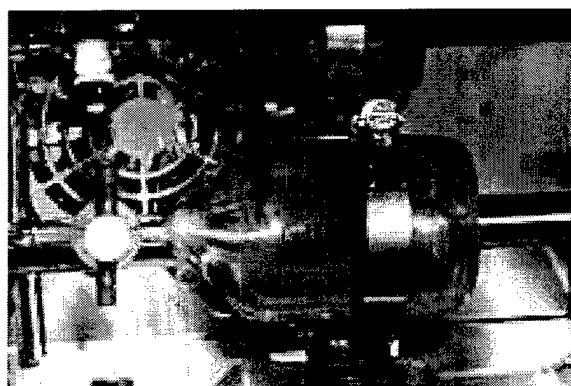


Figure 13. Photograph of a Reaction Flask

All the trials with the storage flasks used the new 35608 microbe. A total of 12 ml of reaction solution were made in the test tubes in which the microbes were centrifuged. The buffer, carboxylic acid and viologen solutions were added to the microbes (in that order) and the mixture shaken to evenly disperse the microbes throughout the solution. Six ml of the mixture was removed from the test tube with a 10-ml Leuer-lock syringe attached to a 6-inch, 20-gauge, stainless-steel needle. The stopcock of the reaction flask was opened and the needle inserted into the flask through the stopcock. The syringe contents were then placed in the flask. After removing the needle and closing the stopcock, the reaction flask was removed from the dry box and reattached to the gas manifold and exit hose (See Figure 14). Argon gas was initially

flowed into the flask and then the gas was switched to carbon monoxide. The exit stopcock was opened to allow the gas to purge through the flask. After 15 minutes, the exit stopcock was closed and the flask pressurized to about 15 psig.

Initially, the flasks were placed on their sides on the shelf of the incubator. The incubator was maintained at 45 °C. The flasks were turned over at 30- minute intervals. This procedure was cumbersome and allowed the mixture to settle in the flasks. To overcome this problem, a Fisher Isotemp Hybridization chamber was purchased. The hybridization chamber is essentially a precision rotisserie (made to hold the hybridization tubes) in a controlled temperature oven. The rotisserie was modified to hold the flasks by removing one set of clamps and replacing these clamps

with 2.5-inch hose clamps. A hose clamp was placed around each flask. A cored foam plug was placed on the stopcock outlet of the flask and inserted into the other set of original clamps that are part of the hybridization chamber (See Figure15). The temperature of the hybridization chamber was set at 45 °C and the rotation speed at 4 rpm. This setup provided both mixing of the solution and good diffusion of the carbon monoxide into the solution. It also provided more accurate temperature control of the reaction vessels.

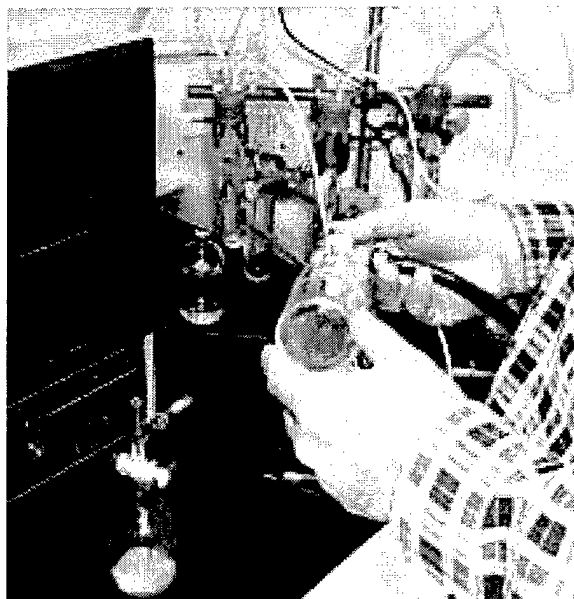


Figure 14. Photograph Showing the Operation of Filling a Reaction Flask with CO



Figure 15. Photograph of Reaction Flasks in Hybridization Chamber

4.4.2 *Experimental Results*

4.4.2.1 *Initial Trials with Gas Purging Setups*

The initial trials with the gas purging setups (the vials or the pear-shaped flasks) used the original 35608 and 39073 microbes purchased from ATCC. The experimental parameters used in these trials and the results are presented in Table VIII. As can be observed from the table, no BUT or BTD were formed in any of these trials. Significant foaming problems were encountered resulting in loss of the reaction solution in the gas stream and/or loss of the microbes in the reaction solution. It became obvious that sparging the CO gas through the reaction solution was not a viable method of contacting the gas with the liquid. Small volume static systems also were not viable because of limited CO/reactant ratios. At the same time, we began to doubt the efficacy of the microbes. Therefore, we decided to try the reaction with n-butyric acid as the substrate. According to the literature, butyric acid is relatively easily reduced to n-butanol. Butyric acid was subjected to the action of both 35608 and 39073 using BV as the mediator. Only trace amounts of n-butanol were found after 20 hours. Butyric acid and n-butanol were analyzed by GC-FID via direct aqueous injection onto a 30-m X 0.25-mm id capillary column coated with SP-1000 (Supelco, 2431-3). A split/splitless injector with a split ratio of 20:1 was used. The injector and detector temperatures were 230 °C and 250 °C, respectively. The oven temperature was initially held at 80 °C for 2 minutes and then programmed at 10 °C/min to 200 °C.

4.4.2.2 *Trials with Sealed 125-ml Reaction Flasks*

The initial trials of the reduction reaction in the sealed 125-ml flasks used butyric acid as the substrate. These trials utilized both the old 39073 and 35608. The effect of the mediator and the pH on the production of n-butanol was also investigated. As shown in Table VII, a maximum of 155 mg/l of n-butanol was produced by 39073 in 20 hours. This n-butanol production is significantly less than that expected from the literature. At this point, we concluded that the problem was with the microbes and new cultures of 35608 and 39073 were ordered from ATCC. All subsequent experiments were performed using the sealed 125-ml reaction flasks with the new culture of 35608. We decided to utilize butyric acid to determine if the carboxylate reduction with this new microbe actually occurred and to perform an initial optimization of the system. This decision was made because butyric acid/butanol analytical results could be available in one day instead of the three days required to analyze the malic or succinic acids and their respective alcohols.

4.4.2.2.1 *Trials with Butyric and New 35608*

Two trials were made with butyric acid in sealed 125-ml flasks. Both trials were conducted by placing the sealed flasks containing the sample in an incubator at 45 °C. The main difference between the two trials was the frequency of rotation of the flasks. In the first trial, the flasks were rotated every 15 minutes. In the second trial, the flasks were only rotated every hour. These trials evaluated the effect of

time on the reduction reaction using BV as the mediator. The effect of using MV instead of BV was evaluated at 4 hours reaction time. A preliminary study was also conducted to look at the effect of substituting formate for CO. The results achieved for butyric acid are presented in Table IX. These results are also plotted in Figure 16.

The results of the butyric acid studies show several trends.

1. Greater than 56% of the butyric acid was converted to butanol in 6 hours.
2. The reduction of the butyric acid at pH 5.8 is as good or better than that reported by Simon and Gunther (1998) for reduction at pH 5.5.
3. Better results are obtained with more frequent rotation of the flasks.
4. MV appears to give a higher reduction rate than BV.
5. The reduction reaction with formate appears to be significantly slower than with CO.

These results were encouraging. The new 35608 microbe appeared to be actively reducing the carboxylic acid group. It would have been nice to obtain more data on butyric acid to try to further evaluate the reduction under more optimized conditions, however, there was insufficient time left on the project to perform additional work with butyric acid.

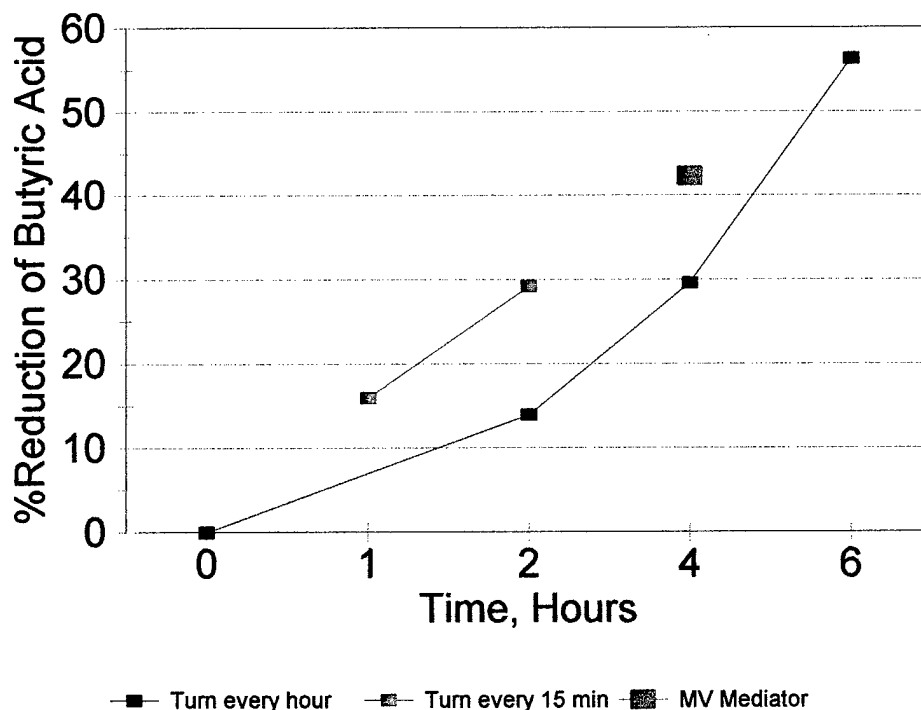


Figure 16. Butyric Acid Reduction with New 35608

Table VIII. Initial Carboxylic Acid Reduction Trials with Old 39073 and 35608

Substrate	pH	Buffer Conc.	Reaction Vessel	Gas Supply	Microbe	Mediator	Rxn. Temp., °C	Rxn. Time, Hrs	Observations	Analytical Results
Malic acid	5.5	.3 M	Vial	CO bubble	39073	MV	40	2, 5 & 20	Most of the solution lost in the gas stream. No blue color.	No BUT formed.
Succinic acid	5.5	.3 M	Vial	CO bubble	35608	MV	40	2, 5 & 20	Most of the solution lost in the gas stream. No blue color.	Not BTD formed.
Malic acid	3.8	.1 M	125-ml vial	CO bubble	35608	MV	40	16	Most of bugs stuck to glass & dry. No blue color.	No BUT formed.
Succinic acid	3.8	.1 M	125-ml vial	CO bubble	35608	MV	40	16	Most of bugs stuck to glass & dry. No blue color.	No BTD formed.
Malic acid	5.5	.1 M	Vial	CO bubble	35608	MV	40	24	Solution turned grayish-blue. Lost significant amount of solution.	No BUT formed.
Succinic acid	5.5	.1 M	Vial	CO bubble	35608	MV	40	24	Solution turned grayish-blue. Lost significant amount of solution.	No BTD formed.
Malic acid	5.8	.1 M	Vial	CO bubble then static	35608	MV	40	24	Solution turned bluish. Bad foaming. Bubbling stopped after 1 hour.	No BUT formed.

Table VIII. Initial Carboxylic Acid Reduction Trials with Old 39073 and 35608

Substrate	pH	Buffer Conc.	Reaction Vessel	Gas Supply	Microbe	Mediator	Rxn. Temp., °C	Rxn. Time, Hrs	Observations	Analytical Results
Succinic acid	5.8	.1 M	Vial	CO bubble then static	35608	MV	40	24	Solution turned bluish. Bad foaming. Bubbling stopped after 1 hour.	No BTD formed.
Malic acid	5.8	.1 M	Vial	CO bubble then static	35608	BV	40	24	Solution turned bluish. Bad foaming. Bubbling stopped after 1 hour.	No BUT formed.
Succinic acid	5.8	.1 M	Vial	CO bubble then static	35608	BV	40	24	Solution turned bluish. Bad foaming. Bubbling stopped after 1 hour.	No BTD formed.
Sodium malate	5.54	.3 M	Vial	CO bubble	35608	MV	43 for 1 hr then 59	24	Solution turned blue. Bad foaming. Bubbler removed and vials placed in shaking water bath @ 59 °C.	No BUT formed.
Sodium succinate	5.54	.3 M	Vial	CO bubble	35608	MV	43 for 1 hr then 59	24	Solution turned blue. Bad foaming. Bubbler removed and vials placed in shaking water bath @ 59 °C.	No BTD formed.

Table VIII. Initial Carboxylic Acid Reduction Trials with Old 39073 and 35608

Substrate	pH	Buffer Conc.	Reaction Vessel	Gas Supply	Microbe	Mediator	Rxn. Temp., °C	Rxn. Time, Hrs	Observations	Analytical Results
Sodium malate	5.41	.1 M	Pear flask	CO bubble	35608	MV	40	24	Solution did not turn blue. Significant dry microbes in ball tube.	No BUT formed.
Sodium malate	5.41	.1 M	Pear flask	CO bubble	35608	BV	40	24	Solution did not turn violet. Significant dry microbes in ball tube.	No BUT formed.
Sodium succinate	5.41	.1 M	Pear flask	CO bubble	35608	MV	40	24	Solution did not turn blue. Significant dry microbes in ball tube.	No BUTD formed.
Butyric acid	5.5	.1 M	Pear flask	CO bubble	35608	BV	40	20	Solution turned deep purple. Significant loss of solution due to bubble over.	Trace amount of butanol formed; significant amount of acetic acid found.
Butyric acid	5.5	.1 M	Pear flask	CO bubble	39073	BV	40	20	Solution turned a bluish purple. Some loss of solution occurred.	Some butanol formed; less acetic acid found.

Table VIII. Initial Carboxylic Acid Reduction Trials with Old 39073 and 35608

Substrate	pH	Buffer Conc.	Reaction Vessel	Gas Supply	Microbe	Mediator	Rxn. Temp., °C	Rxn. Time, Hrs	Observations	Analytical Results
Butyric acid	5.8	.1 M	125-ml sealed flask	Static, ~ 2 atm	35608	BV	40	20	Solution turned purple	52.1 ppm butanol formed
Butyric acid	5.8	.1 M	125-ml sealed flask	Static, ~ 2 atm	35608	MV	40	20	Solution turned blue	47.7 ppm butanol formed
Butyric acid	5.38	.1 M	125-ml sealed flask	Static, ~ 2 atm	35608	BV	40	20	Solution turned purple	34.6 ppm butanol formed
Butyric acid	5.38	.1 M	125-ml sealed flask	Static, ~ 2 atm	35608	MV	40	20	Solution turned blue	18.0 ppm butanol formed
Butyric acid	5.8	.1 M	125-ml sealed flask	Static, ~ 2 atm	39073	BV	40	20	Solution turned purple	155.0 ppm butanol formed
Butyric acid		.1 M	125-ml sealed flask	Static, ~ 2 atm	39073	MV	40	20	Solution turned blue	54.4 ppm butanol formed
Butyric acid		.1 M	125-ml sealed flask	Static, ~ 2 atm	39073	BV	40	20	Solution turned purple	154.1 ppm butanol formed
Butyric acid		.1 M	125-ml sealed flask	Static, ~ 2 atm	39073	MV	40	20	Solution turned blue	53.0 ppm butanol formed

Table IX. Butyric Acid Reduction with New 35608 at pH 5.8 and 45°C

Initial Butyric acid Conc., mM	Reducing Agent	Mediator	Reaction Time, hr	Turn Rate, min.	Final Butanol Conc., mM	% Reduction
39.65	CO	BV	1	15	6.32	15.95
39.65	CO	BV	2	15	11.6	29.14
35.00	CO	BV	2	60	4.9	13.93
35.00	CO	BV	4	60	10.3	29.52
35.00	CO	BV	6	60	19.7	56.33
35.00	CO	MV	4	60	14.8	42.29
39.65	Formate	BV	1	15	1.08	2.73

4.4.2.2.2 *Trials with Succinic Acid and New 35608*

The next set of experiments briefly evaluated the ability of the new 35608 microbe to reduce succinic acid to 1,4-butanediol. The conditions and the results obtained are presented in Table X. The chromatograms of the reacted samples at T=3.5 or 4 hours showed the presence of a significant quantity of the lactone.

Table X. Succinic Acid Reduction with New 35608 at 45 °C

Initial Succinic Acid Conc., mM	pH	Mediator	Reaction Time, hr	Turn Rate, min.	Lactone Conc., mM	Final Conc. of BTD, mM	% Reduction to BTD
19.97	5.96	BV	3.5	30	0.855	0.22	1.08
22.82	5.69	BV	3.5	30	1.42	0.38	1.42
21.04	5.2	BV	3.5	30	2.87	0.86	4.09
28.16	5.26	MV	4.0	30	0.80	0.56	1.99

Several interesting observations can be made from the table. The reaction rate is definitely affected by the pH of the solution with faster rates observed at lower pH values. It appears that the optimal pH for this reduction reaction is probably <5, however, additional studies are required to determine this optimal pH. These preliminary results seem to indicate that BV is a better mediator for this reduction reaction than MV. Significantly more lactone and diol are formed with BV as the

mediator. The lactone to BTB ratio with BV ranges from 3.886/1 for the higher pH to 3.337/1 for the lower pH. In contrast, the lactone to BTB ratio for MV is 1.429/1. The BV reduction reaction also yields approximately twice the BTB product as the MV reduction reaction at approximately the same pH and time.

It is difficult to compare the reduction of succinic acid to BTB with the data given in the literature because of the way Simon and coworkers [Simon and Gunther, 1998; Huber *et al.*, 1995; Simon *et al.*, 1987] presented their data. In one paper, they report the PN is for the diol (corresponding to a 0.3% reduction in 1 hour) while in another paper they report the same PN is for one pair of electron consumed (presumably the lactone). Whatever the case, it appears that our reduction of succinic acid to BTB is occurring at least as fast or much faster than the literature values.

It would have been very productive to further optimize the reduction reaction for succinic acid, however, at this point we were into the sixth month of the six month project. Therefore, we decided to move on and try the reduction with malic acid.

4.4.2.2.3 Trials with Malic Acid and New 35608

A set of experiments was run to try to determine the conditions under which malic acid could be reduced to BUT by *Clostridium thermoaceticum*. These studies used an initial malic acid concentration of 22 mM. The conditions for each of these trials and the results are presented in Table XI. Trace amounts of BUT were detected in three samples, 8-1, 8-4 and 9-3. All these samples had a pH of <5.0 and used microbes that were just entering the resting stage, *i.e.*, ~24 hours old. Trace results were obtained when both MV and BV were used as the mediator. However, not enough data on BV were obtained to draw any conclusions.

In many of these experiments, malic acid in the reaction samples was less than in the T=0 samples, indicating the disappearance of malic acid. Several possibilities exist to explain the malic acid disappearance without the concurrent appearance of one of the lactones, maleic acid, or BUT, *i.e.*,

- Malic acid could be utilized by the cells,
- BUT produced could be incorporated into the cellular materials or utilized by the cells, or
- AHL or BHL produced could be incorporated into the cellular materials or utilized by the cells.

To try to determine if BUT was incorporated into the cells, sample # 9-4 was spiked with BUT to a level of 174.4 mg/l before the sample was divided. Thus, both the T=0 and T=4 samples contained an equivalent amount of BUT. Recovery of this spike was 83.8% for the T=0 sample and 89.5% for the T=4 sample, thus, indicating that BUT is not incorporated into the cellular material or used by the cells. AHL was similarly spiked into sample 11-4 to give 180.3 mg/l solution. Recovery of the AHL was only 19.9% in

the T=0 sample and 18.5% in the T=4 sample. This recovery was significantly less than expected from the QC samples and indicates that AHL may be bound to the cellular material. The only methodology available to confirm these suspicions is with ^{14}C tracer studies. Similarly, the only way to confirm if malic acid is being used by the cells is through ^{14}C studies.

Preliminary results suggests that some combination of reaction conditions could result in efficient production of BUT. However, due to time limitations on the project, further work on optimizing the process for BUT production was not performed. The following process parameters need to be optimized before any firm conclusions on the microbial production of BUT from malic acid can be made:

- pH,
- concentration of microbes,
- age of microbes,
- mediator,
- reaction time,
- reaction temperature,
- reaction atmosphere components, and
- reaction atmosphere pressure.

4.5 Task 5 - Effect of Ionic Liquid on 1,2,4-Butanetriol Production

Two ionic liquids were ordered and received, 1-butyl-3-methylimidazolium hexafluorophosphate (Acros 354170250) $[\text{C}_4\text{mim}][\text{PF}_6]$ and 1-butyl-3-methylimidazolium chloride (Acros 354090250) $[\text{C}_4\text{mim}]\text{Cl}$. $[\text{C}_4\text{mim}][\text{PF}_6]$ is a liquid at room temperature and is immiscible with water. This ionic liquid would be the solvent of choice for the microbial reduction reaction if the BUT product is soluble. $[\text{C}_4\text{mim}]\text{Cl}$ is a solid at room temperature and is the starting material for synthesis of other ionic liquids not commercially available.

To determine if BUT and malic acid were soluble in $[\text{C}_4\text{mim}][\text{PF}_6]$, 0.03595 grams of *d,l*-malic acid were added to 3.83548 grams of $[\text{C}_4\text{mim}][\text{PF}_6]$ in a 100-mm X 13-mm screw-capped test tube. BUT (0.05263 grams) was added to 3.88633 grams of $[\text{C}_4\text{mim}][\text{PF}_6]$ in a second test tube. The mixtures were vortexed to disperse the liquid or solid in the ionic liquid. Neither the BUT nor the malic acid appeared to dissolve after standing at room temperature for 24 hours. The malic acid had crystals in the bottom of the test tube and the BUT solution was very cloudy. The test tubes were placed in the incubator at 65 °C for 24 hours. Some of the BUT appeared to dissolve as indicated by the disappearance of the cloudiness of the solution. The malic acid did not dissolve. Because of the problems associated with Task 4, the ionic liquid as a reaction medium for the reduction of malic acid was not further pursued.

Table XI. Malic Acid Reductions with New 35608 at 45 °C

#	pH	Age of Microbes State*	Amount of Microbes, ml	Mediator	Reaction Time, hr	Turn Rate, min.	Results
6-2	5.12	24 NF	3	MV	4.0	30	No BUT
6-3	4.92	24 NF	3	MV	4.0	30	No BUT
6-4	4.69	24 NF	3	MV	4.0	30	No BUT
8-1	4.6	24 NF	3	BV	25.5	.25	Trace BUT, AHL & BHL
8-2	4.57	24 NF	3	MV	25.5	.25	No BUT
8-4	4.61	24 NF	3	MV	25.5	.25	Trace BUT, AHL & BHL
9-1	4.90	23.25 NF	5	MV	4.0	.25	No BUT
9-2	4.99	23.25 NF	4.5	MV	4.0	.25	No BUT
9-3	4.82	23.25 F	5	MV	4.0	.25	Trace BUT
9-4	4.91	23.25 NF	5	MV	4.0	.25	83.8 % of BUT spike was recovered in T=0, 89.5% in T=4
10-1	4.65	32 NF	3	MV	4.0	.25	No BUT
10-2	5.10	32 NF	3	MV	4.0	.25	No BUT
10-3	4.63	46 NF	3	MV	4.0	.25	No BUT
11-1	4.63	46 NF	3	MV	4.0	.25	No BUT
11-2	4.62	46 F	3	MV	4.0	.25	No BUT
11-3	4.63	32 F	3	MV	4.0	.25	No BUT
11-4	4.67	46 NF	3	MV	4.0	.25	No BUT, 19.9 % of AHL spike was recovered in T=0, 18.5% in T=4

4.6 Task 7 - Preliminary Design of a Continuous Bench-scale 1,2,4-Butanetriol Production Process

The development of an efficient and cost-effective microbial process to synthesize BUT from malic acid, requires that the growth of the microbes be accomplished in a continuous reactor utilizing an inexpensive media while still maintaining the activity of the enzymes. A drawing of a continuous fermenter to grow *Clostridium thermoaceticum* in the laboratory is shown in Figure 17. This fermenter consists of a 6000-ml Bioreactor (e.g., Kontes Brand Cytostir) that has been modified to provide for the anaerobic conditions necessary for the growth of *Clostridium thermoaceticum*. The plastic caps have been replaced with butyl rubber stoppers. To preserve the anaerobic conditions, all inlet and outlet tubing should be stainless-steel or C-flex or equivalent. The carbon dioxide gas is provided to the reactor via a sparger. pH control of the medium is provided by a pH electrode, pH controller and base addition pump. Fresh medium is continually pumped into the reactor. Medium is pumped out of the reactor through a cross flow filter. The permeate, containing acetic acid, is drawn off the filter and the retentate is recycled back to the reactor. Cells are also continuously pumped out of the reactor. The recycle ratio, medium feed, and cell purge rates must be determined experimentally to provide cells in the proper phase of growth for the reduction reaction.

The medium required to grow *Clostridium thermoaceticum* will be a major contributor to the cost of the BUT synthesis. Lundie and Drake (1984) developed a minimally defined medium for the growth of *Clostridium thermoaceticum* while still maintaining enzyme activity. However, they did not evaluate the effects of this medium on all the enzymes used in carboxylic acid reduction. The specific growth requirements necessary to optimize the production of these enzymes will have to be defined. *Clostridium thermoaceticum* is found in nature in manure and composts. It would be more cost-effective if a waste material, e.g., chicken manure, could be used to supply the major nutrients with only the addition of specific compounds to maximize the production of the desired enzymes.

The actual reduction reaction is probably best accomplished in a tricking filter or biodisc type of system. Both of these systems provide good diffusion of gas into the liquid medium. For the laboratory systems, a tricking filter arrangement will be the easiest to construct (See Figure 18). The microbes can be immobilized in a variety of media including K-carrageenan (Simon and Lebertz (1989), polysacchrides, cellulose agarose, gelatin, cellulose acetate, silica gel, polyacrylamide, etc. (Hideo and Wataru (1996). The immobilized microbes can then be coated onto a support. The carboxylic acid solution is trickled down the filter with the reduction gas flowing up the filter. The exit gas is scrubbed with sodium hydroxide to remove the carbon dioxide produced in the reaction, and the gas recycled through the filter. A portion of the malic acid liquid is recycled through the filter to obtain maximum reduction. The alcohol product is collected from the bottom of the filter.

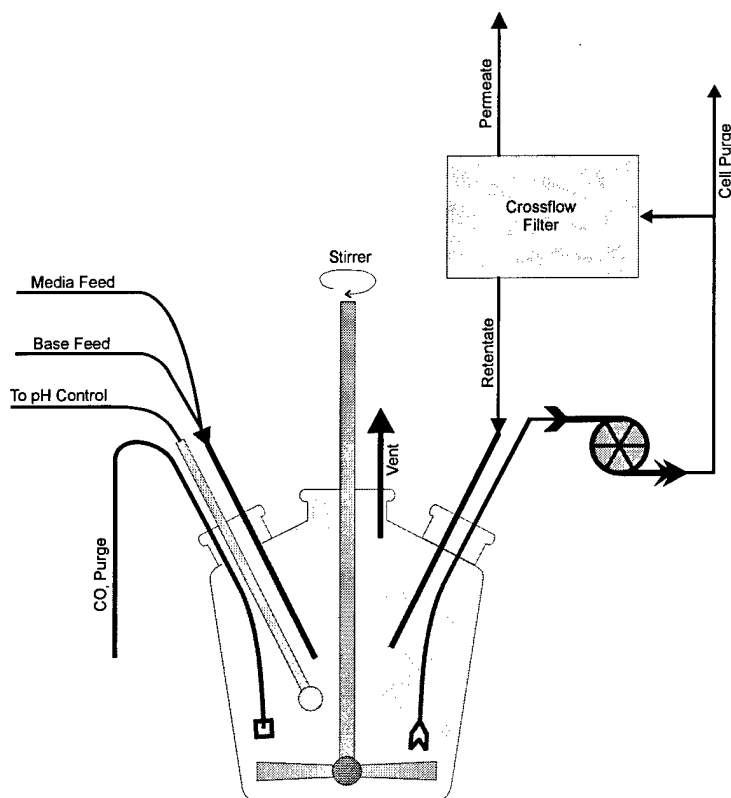
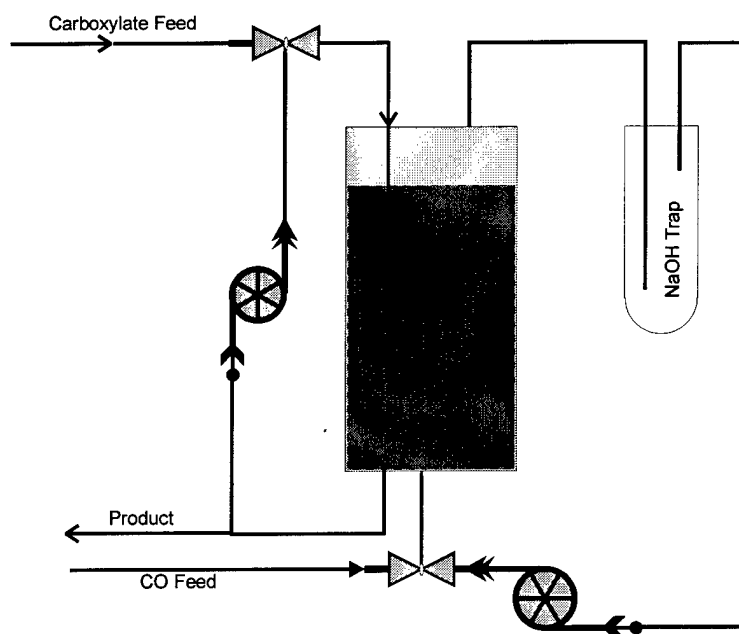


Figure 17. Diagram of a Laboratory Flow-through Fermenter

Figure 18. Laboratory Trickle Filter Reactor



5. Conclusions and Recommendations

The various experimental difficulties encountered during this project prevented us from obtaining our goal of demonstrating microbial production of 1,2,4-butanetriol from malic acid. Only trace amounts of 1,2,4-butanetriol were made by this process, however, the experimental conditions have not yet been optimized to produce 1,2,4-butanetriol. The generalized reaction conditions used by Simon and Gunther (1998) were found to be inappropriate for reductions of complex carboxylic acids such as malic acid and even succinic acid. The experimental conditions for the microbial reduction of carboxylic acids to the corresponding alcohols were found to be very dependent on the carboxylic acid. The optimal pH for the reaction tends to shift with the pK_a of the acid. The optimal mediator(s) for the reaction also appears to be dependent on the carboxylic acid although not enough data are available to identify any trends. It is also expected that the optimal reaction temperature and substrate concentration are also a function of the carboxylic acid.

Data obtained for the reduction of butyric acid indicate that 56% of the butyric acid was converted to n-butanol within 6 hours under less than optimal conditions. The reduction of succinic acid was slower than that observed for butyric acid, however, significantly more work remains to optimize the experimental conditions for this reaction. Only preliminary data were obtained on malic acid reduction. These data indicate that 1,2,4-butanetriol can be produced from malic acid, however, the experimental conditions employed were far from optimal. Thus, a significant amount of work remains to be performed before the efficiency and cost-effectiveness of this process can be ascertained.

Work that still needs to be performed to assess the value of this process for producing 1,2,4-butanetriol includes the following:

- Determine the optimum pH of the reaction at a given temperature with MV and BV as mediators.
- Determine the effect of temperature on the reaction at the optimal pH with both MV and BV as mediators.
- Determine the effects of buffer concentration and type of buffer at the optimum pH, temperature, mediator combination.
- Determine the effect of different atmospheres and pressures at the optimum pH, temperature, mediator and buffer combination.
- Determine the rate constant for the reaction under the optimized conditions of pH, temperature, mediator, buffer, atmosphere components and atmosphere pressure.

Carboxylic acid reduction reactions with *Clostridium thermoaceticum* have wide-reaching synthetic potential. We are continuing this research in our laboratories and will provide further results to the Navy technical personnel as they become available.

6. References

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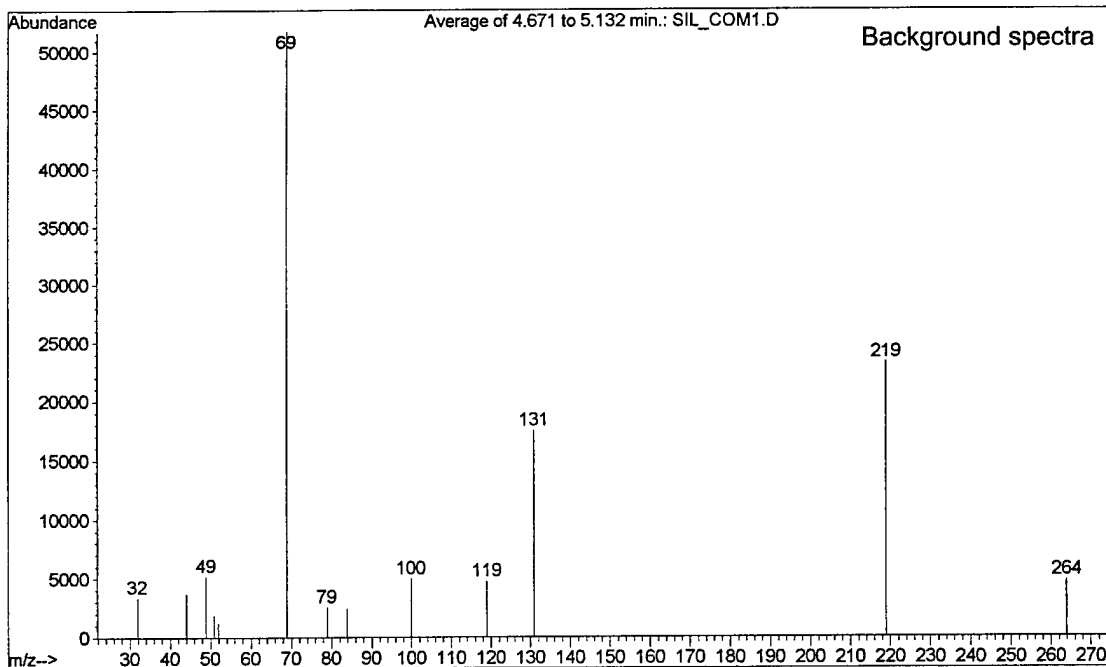
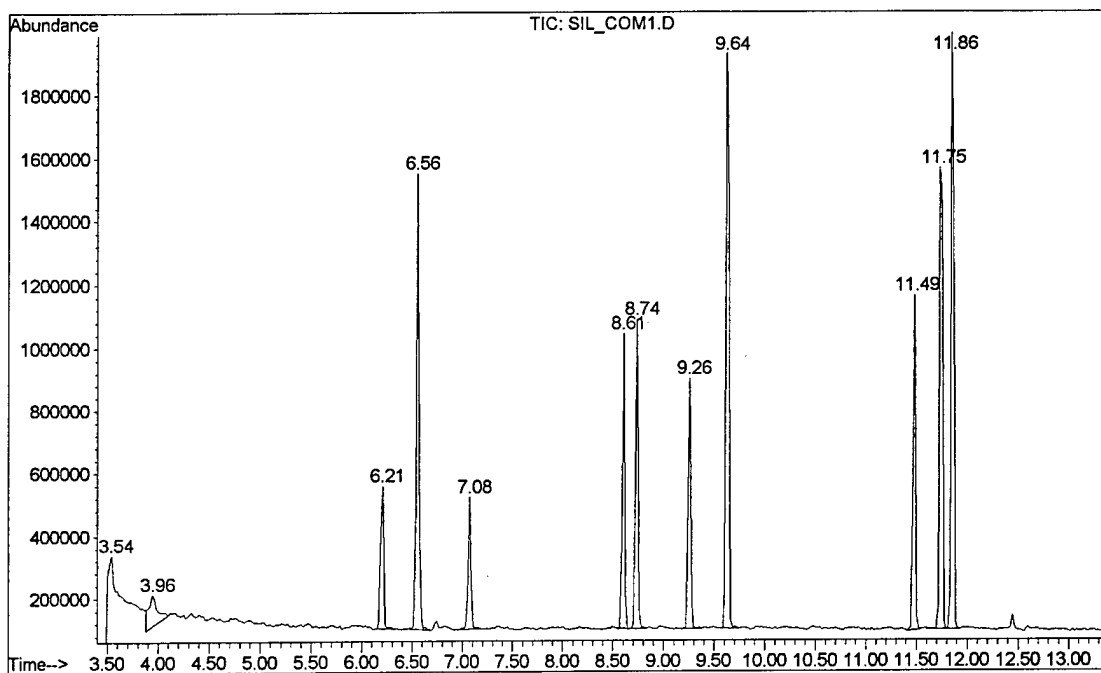
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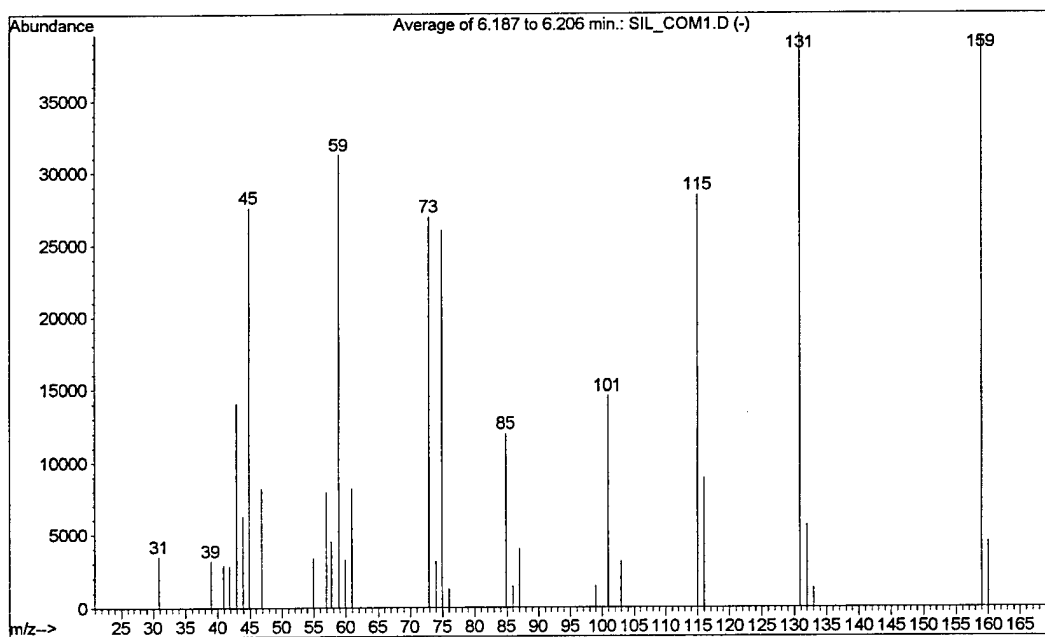
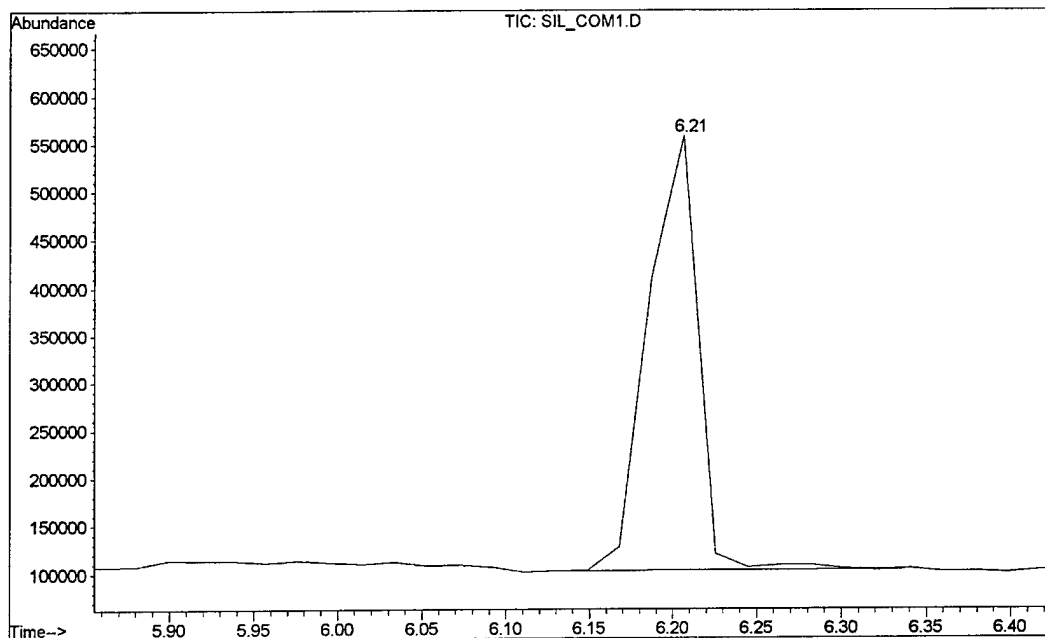
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Appendix A
GC-MS of Analytes

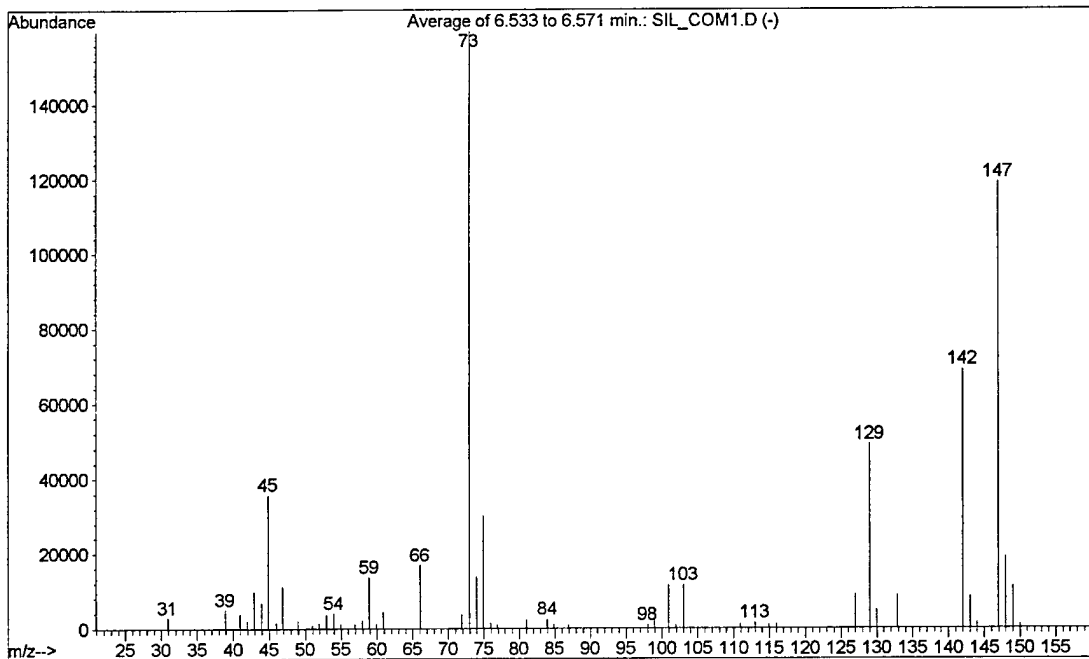
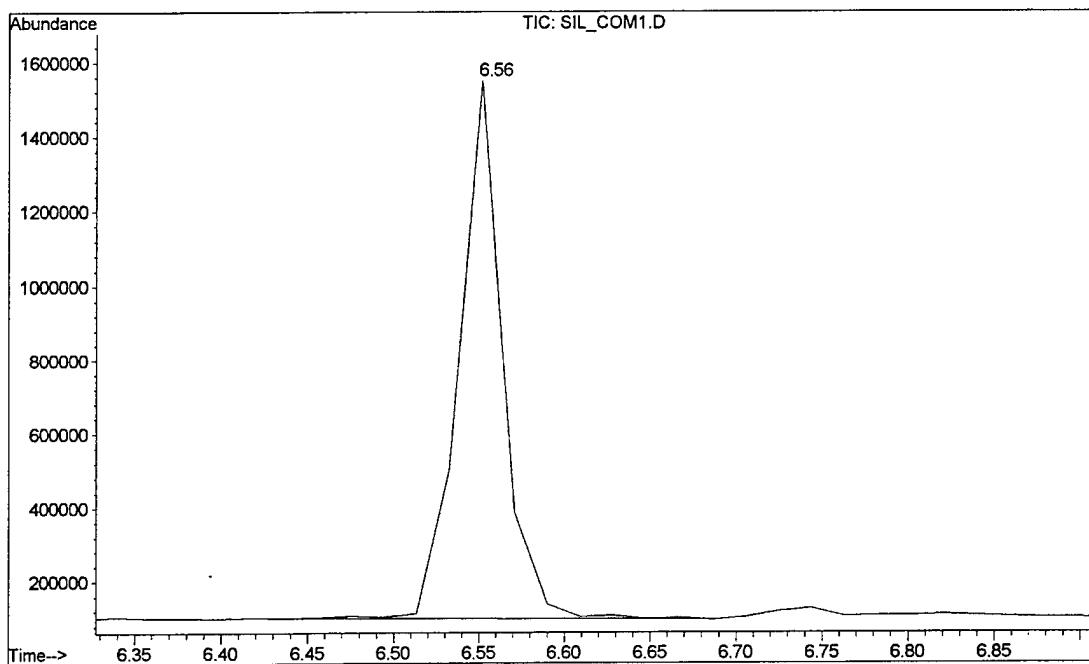
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Misc Info : ~40ppm each component From Silylation Reaction mix
Vial Number: 0



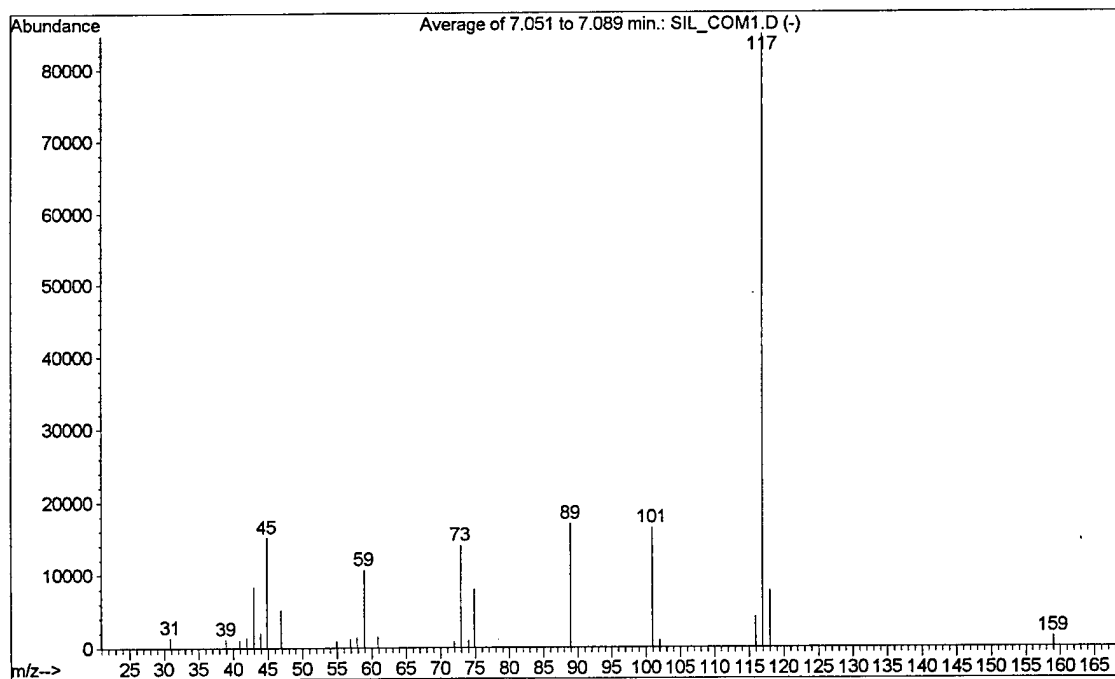
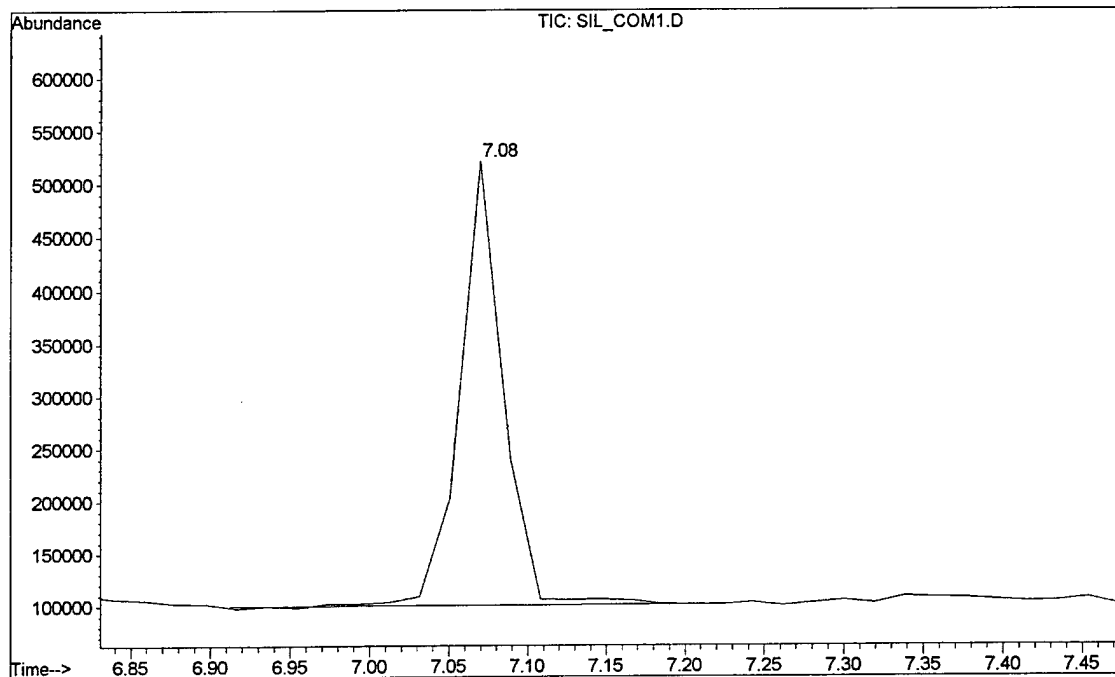
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Vial Number: 0



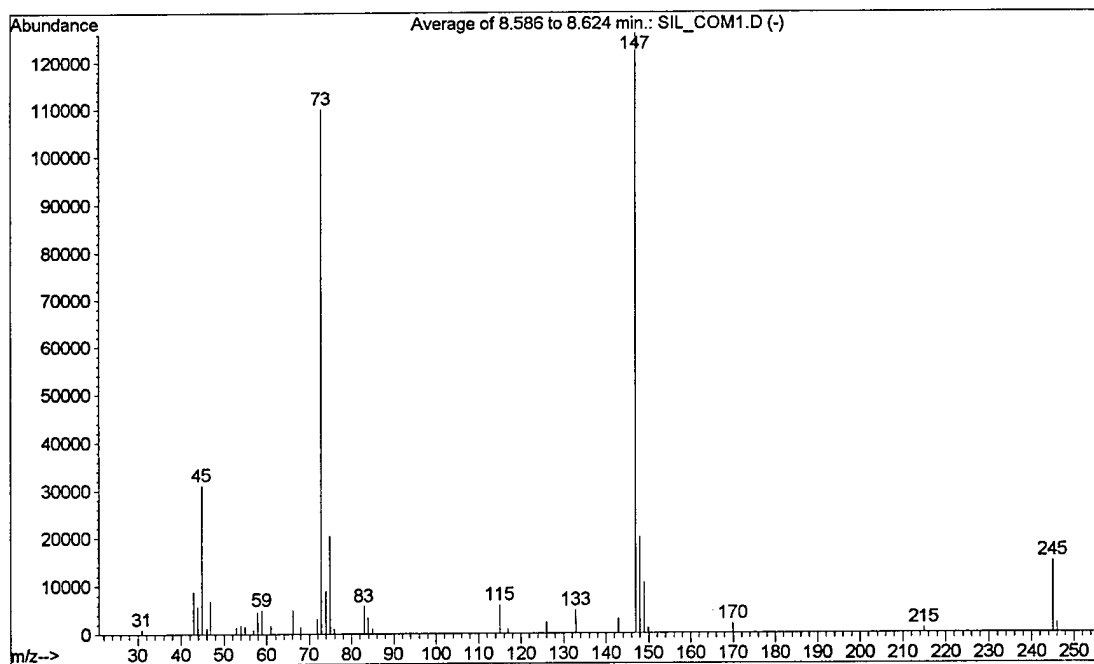
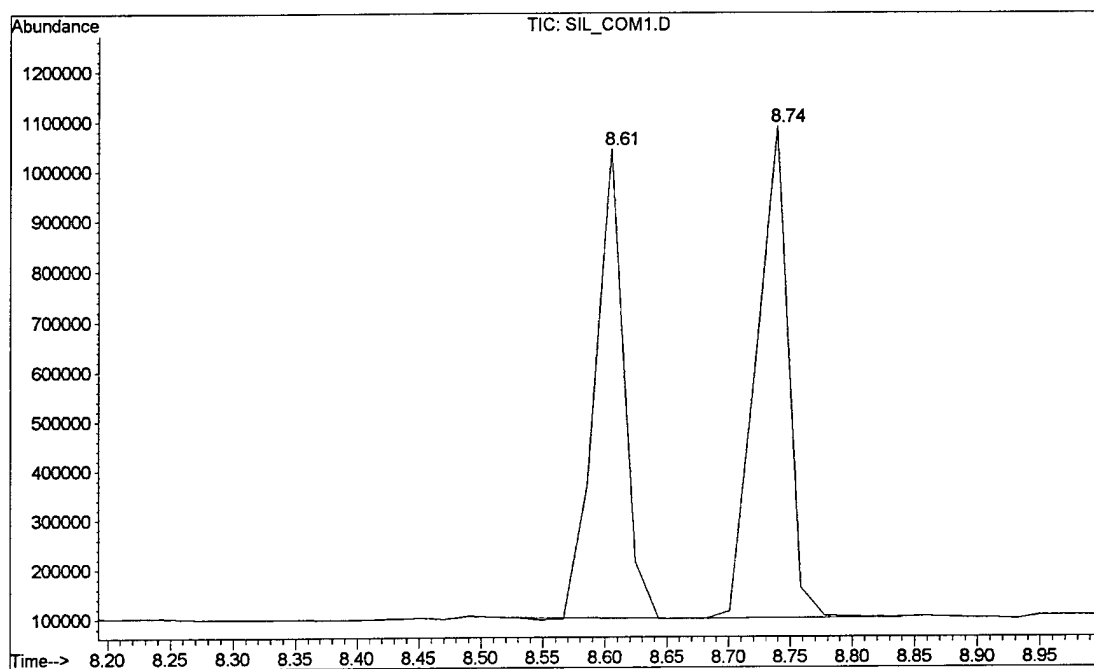
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Vial Number: 0



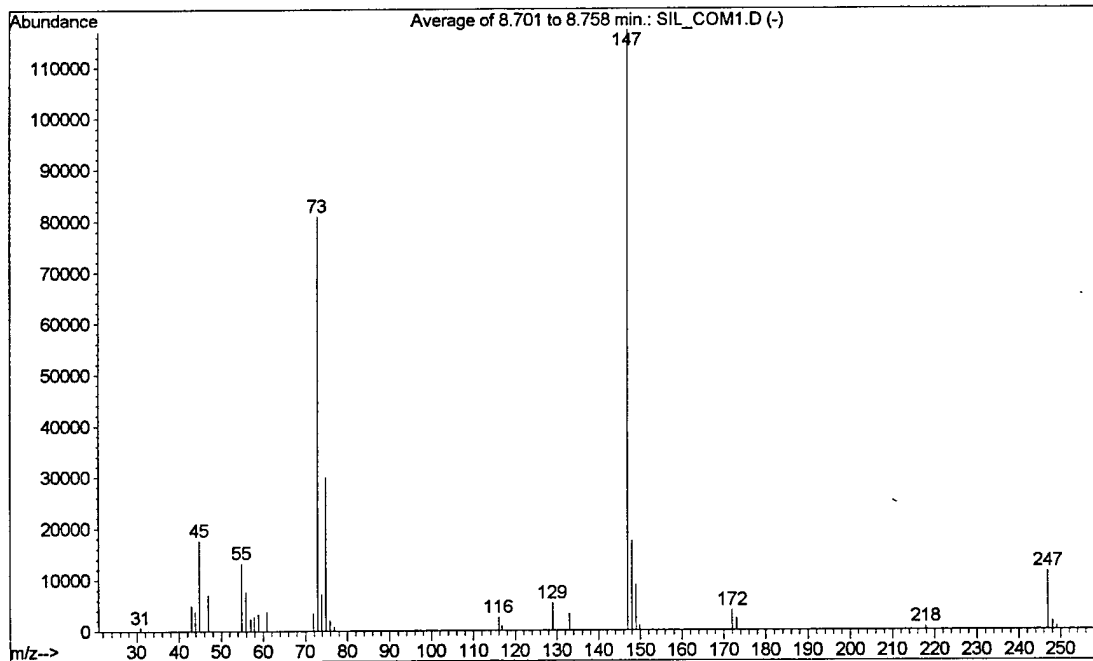
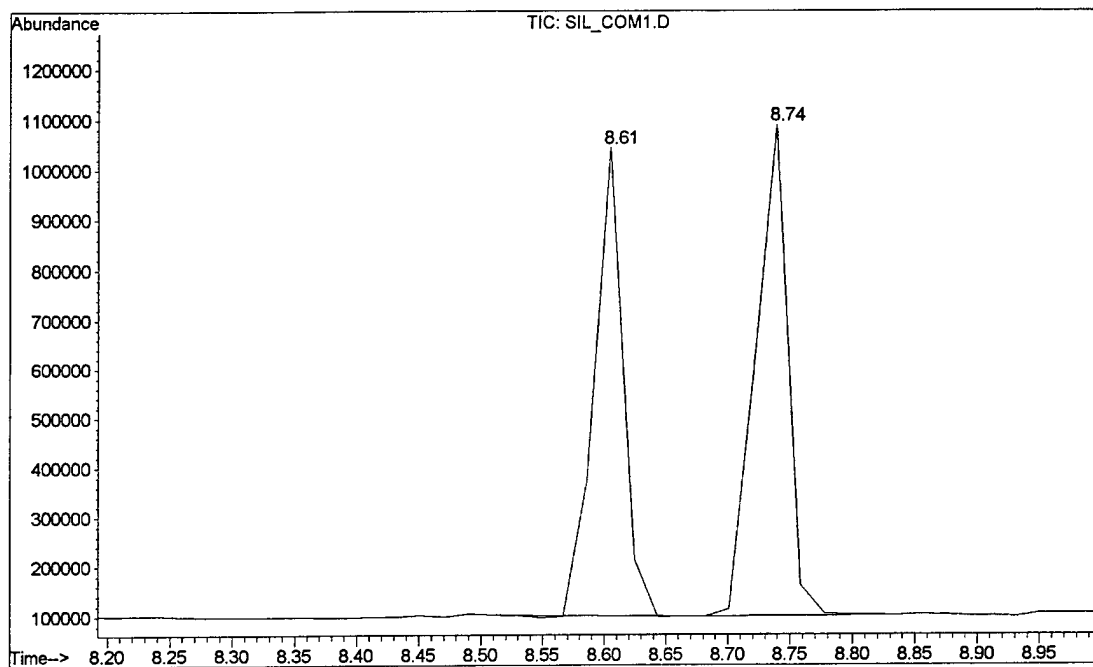
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Vial Number: 0



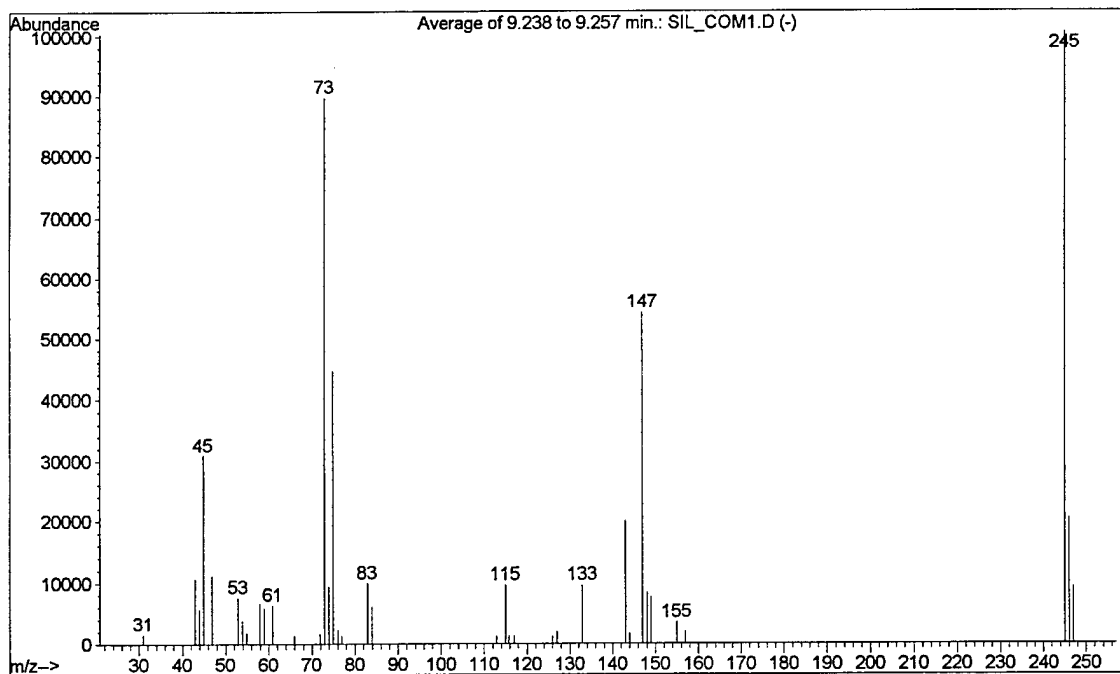
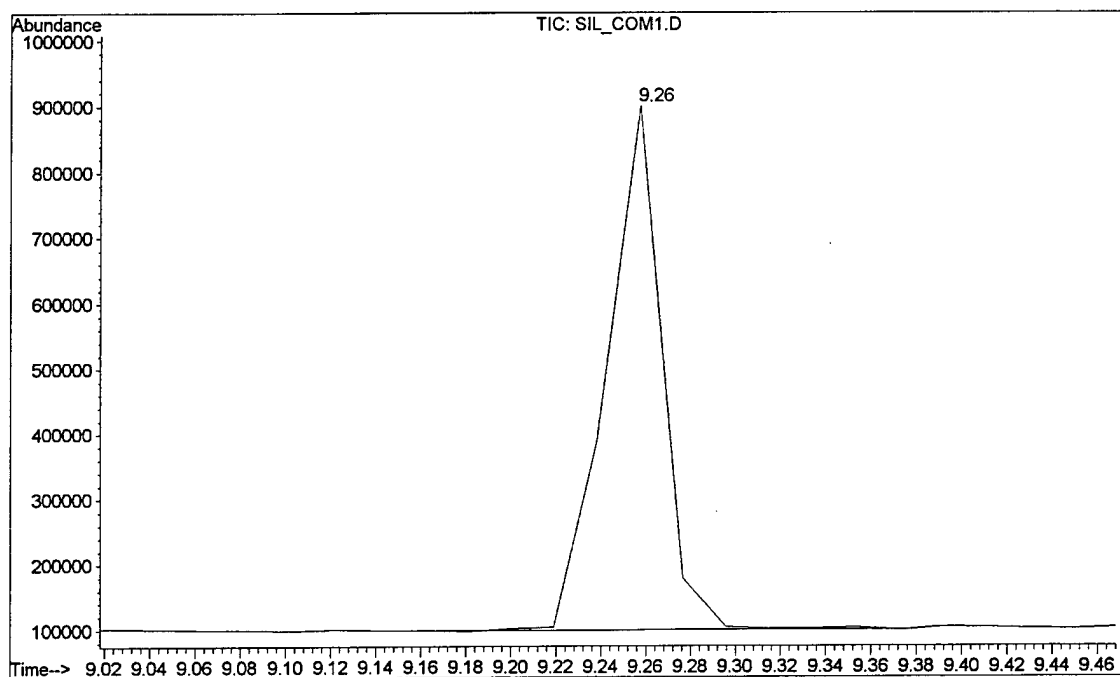
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Vial Number: 0



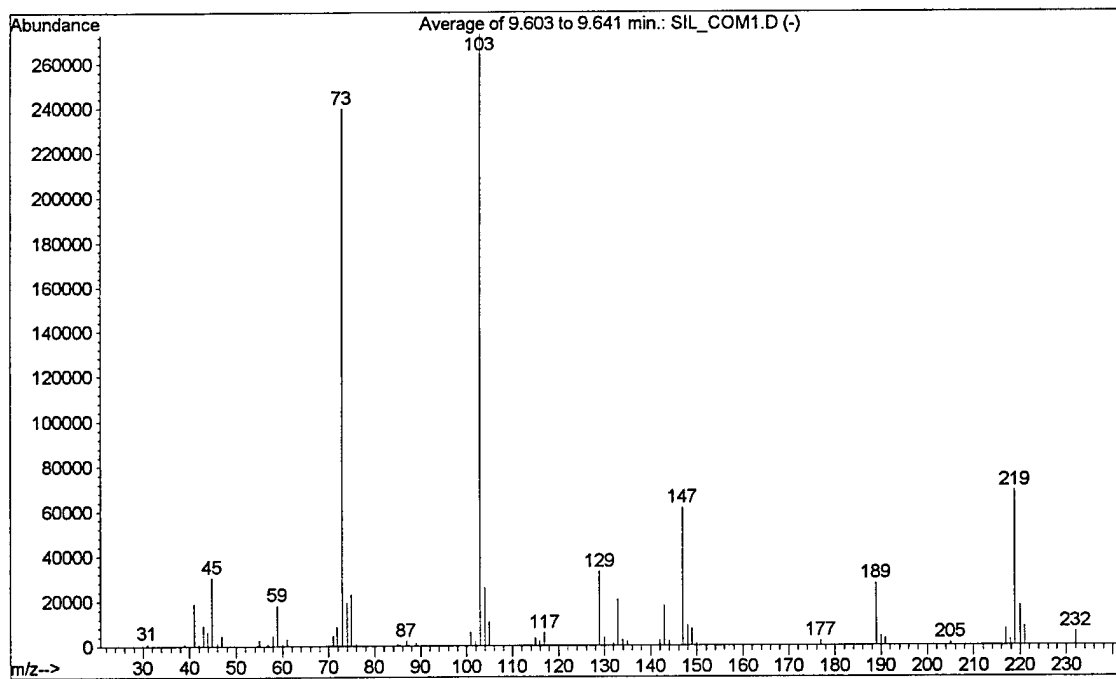
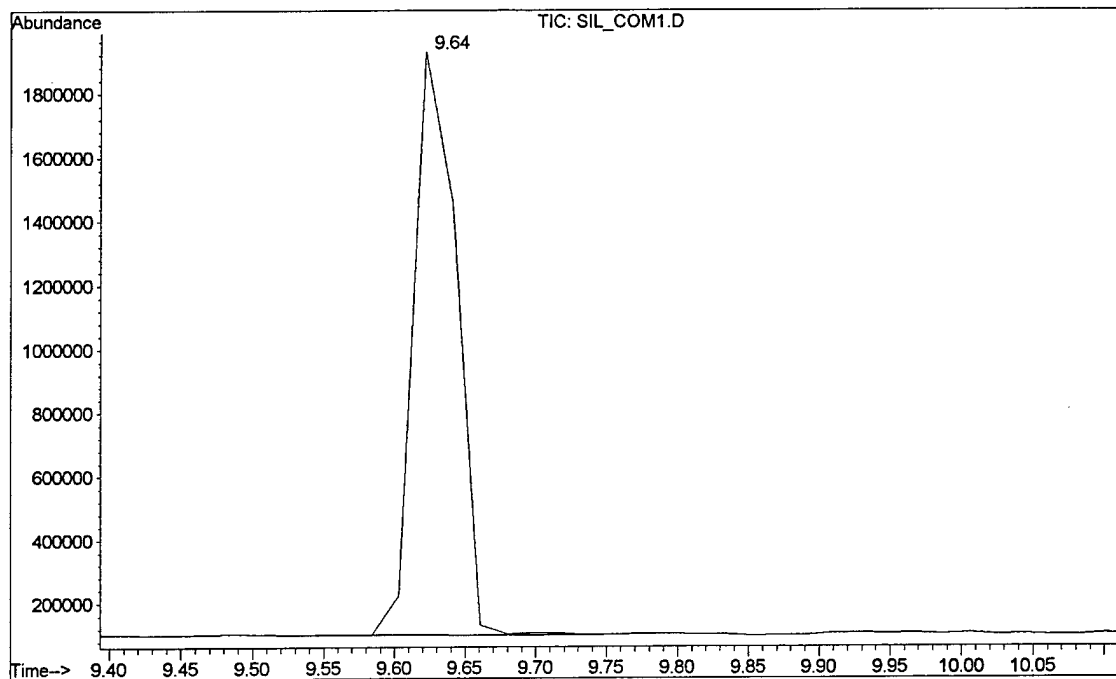
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Vial Number: 0



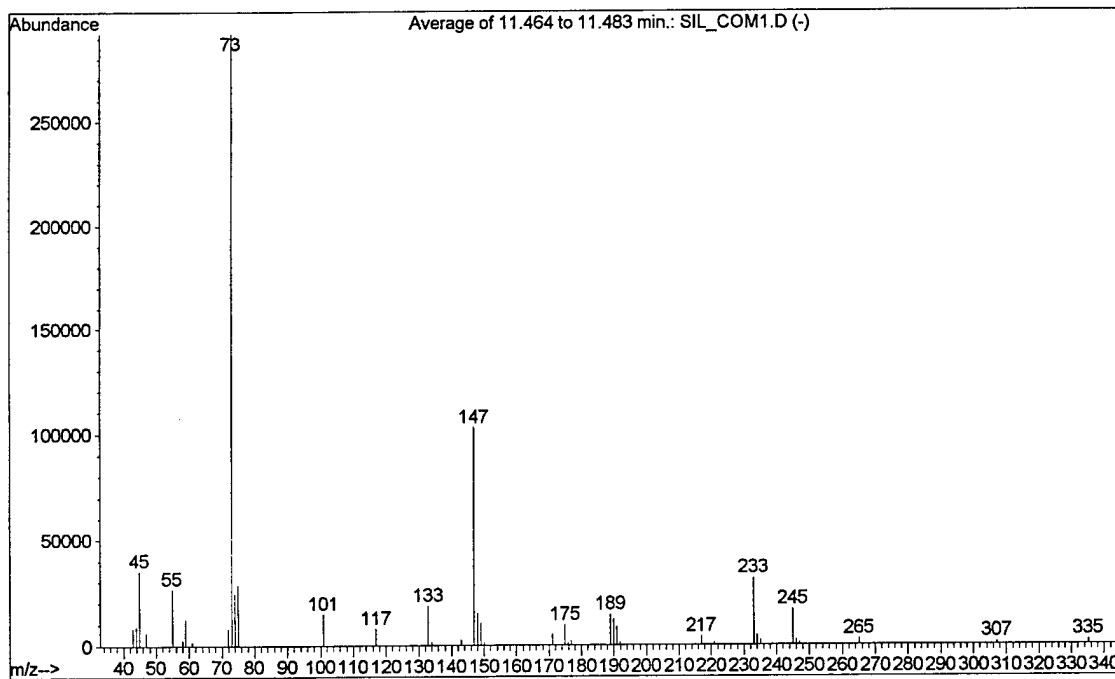
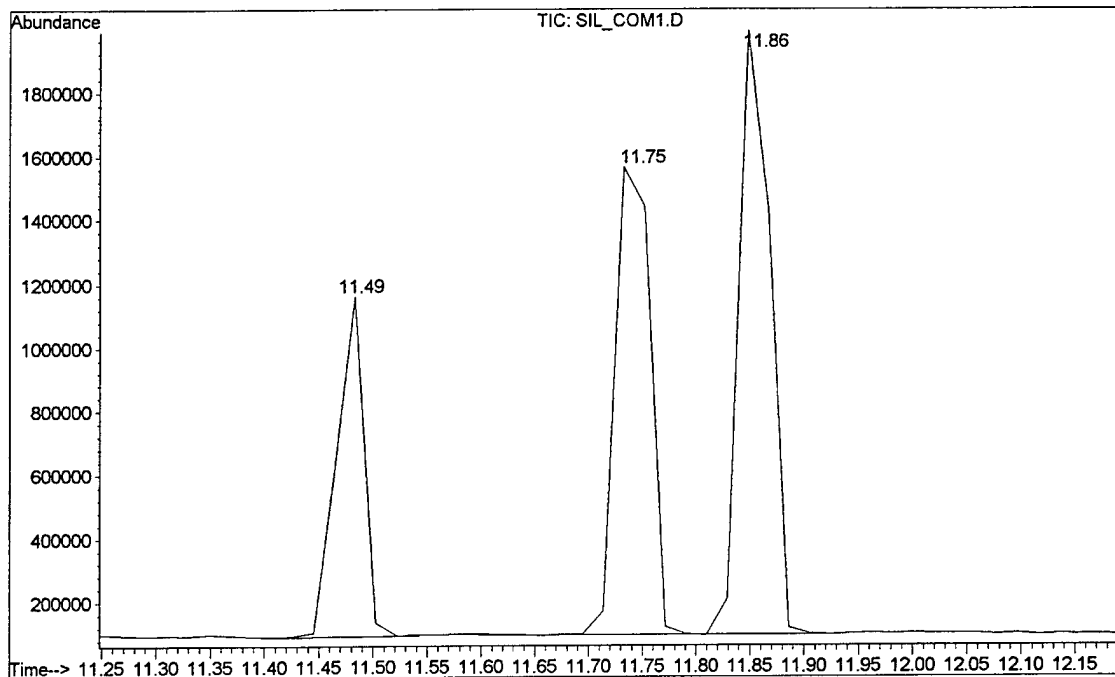
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Vial Number: 0



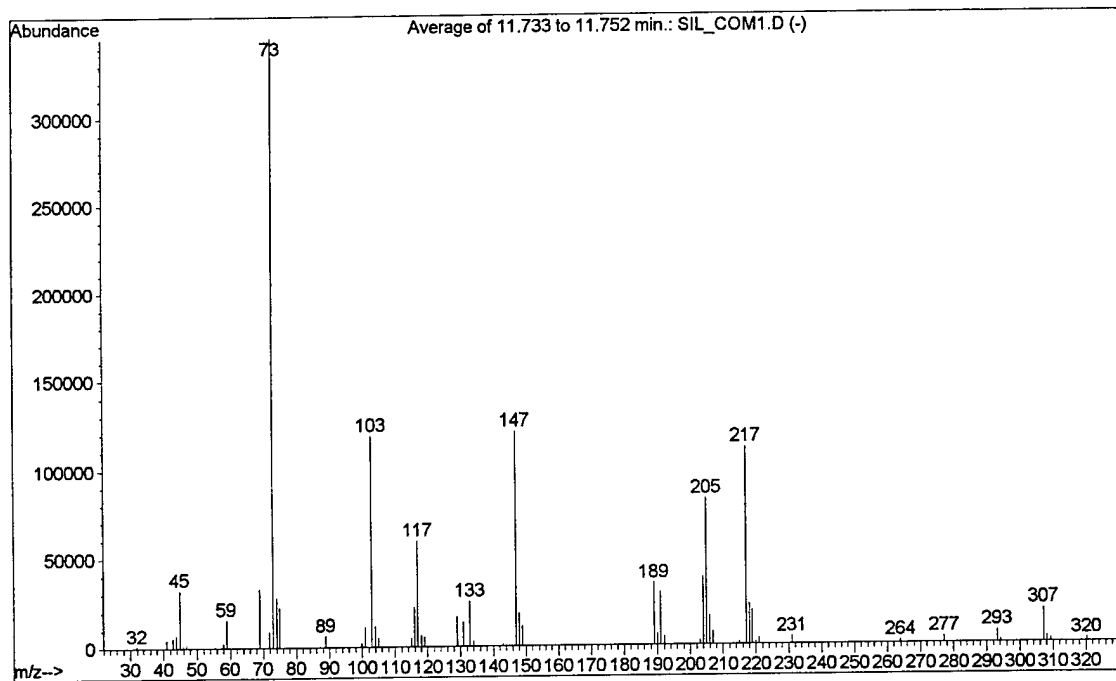
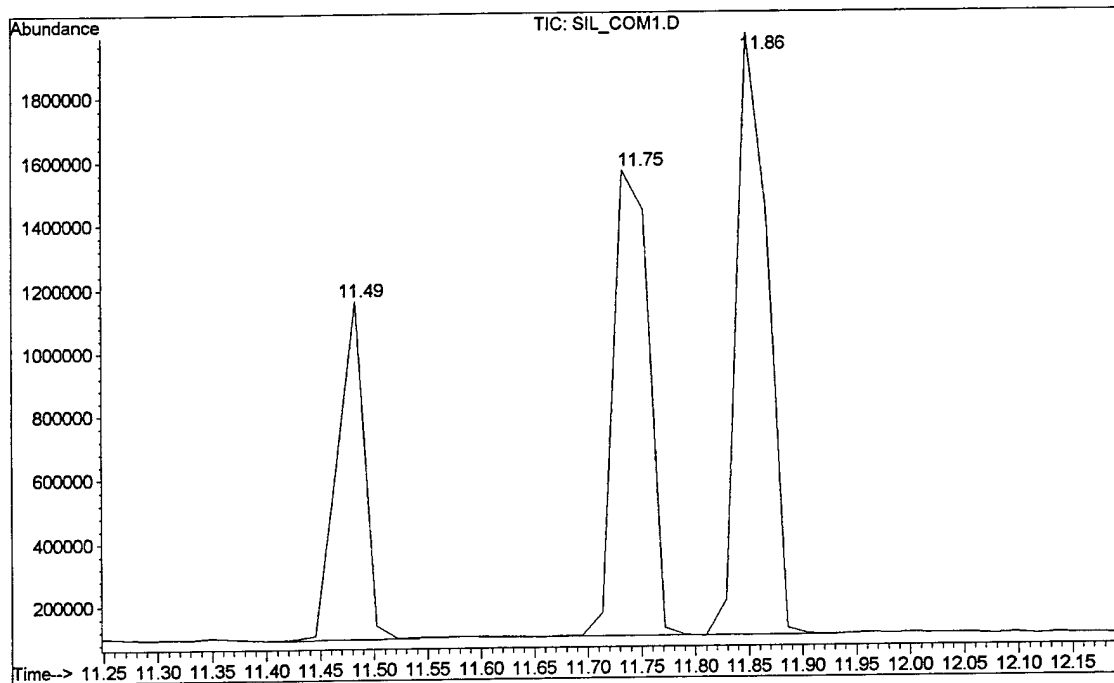
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Vial Number: 0



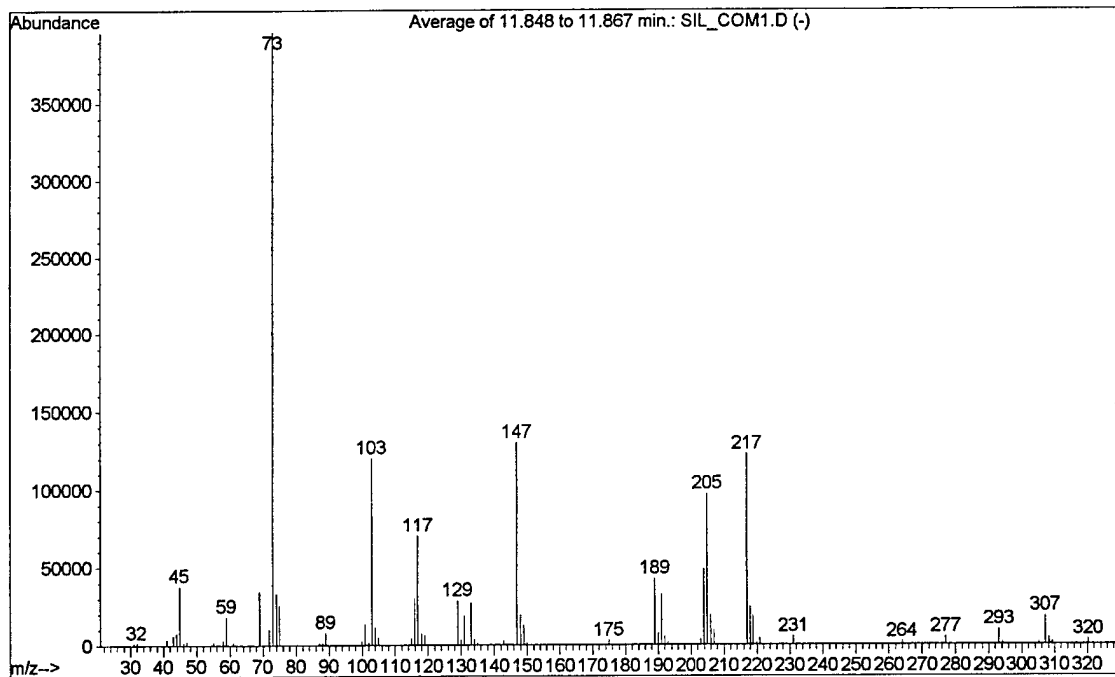
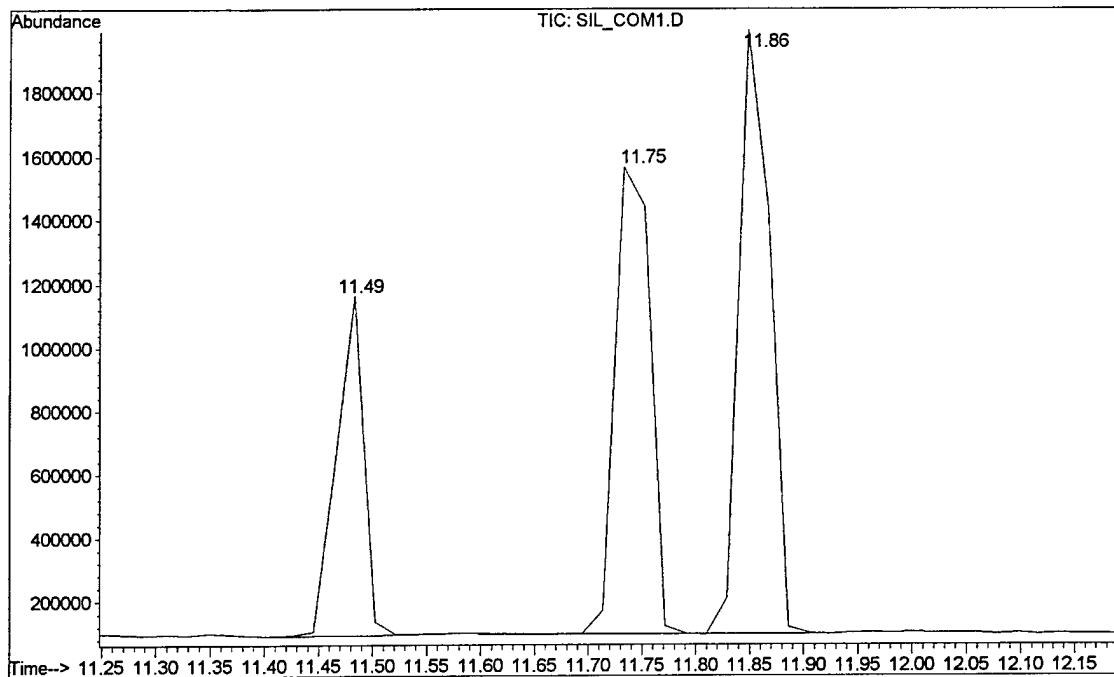
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Vial Number: 0



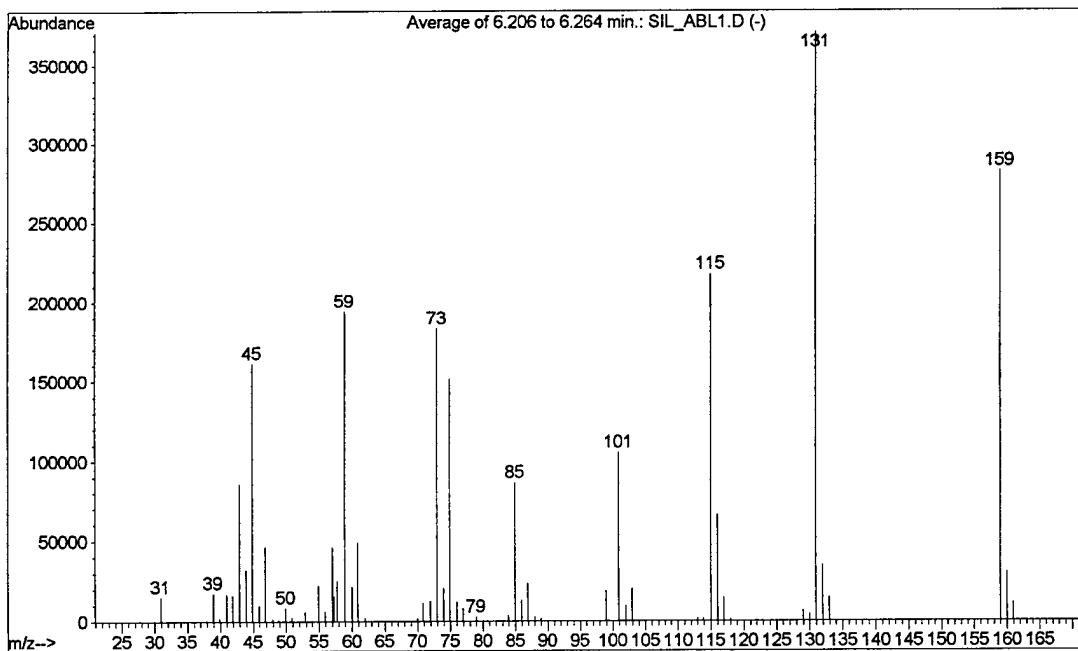
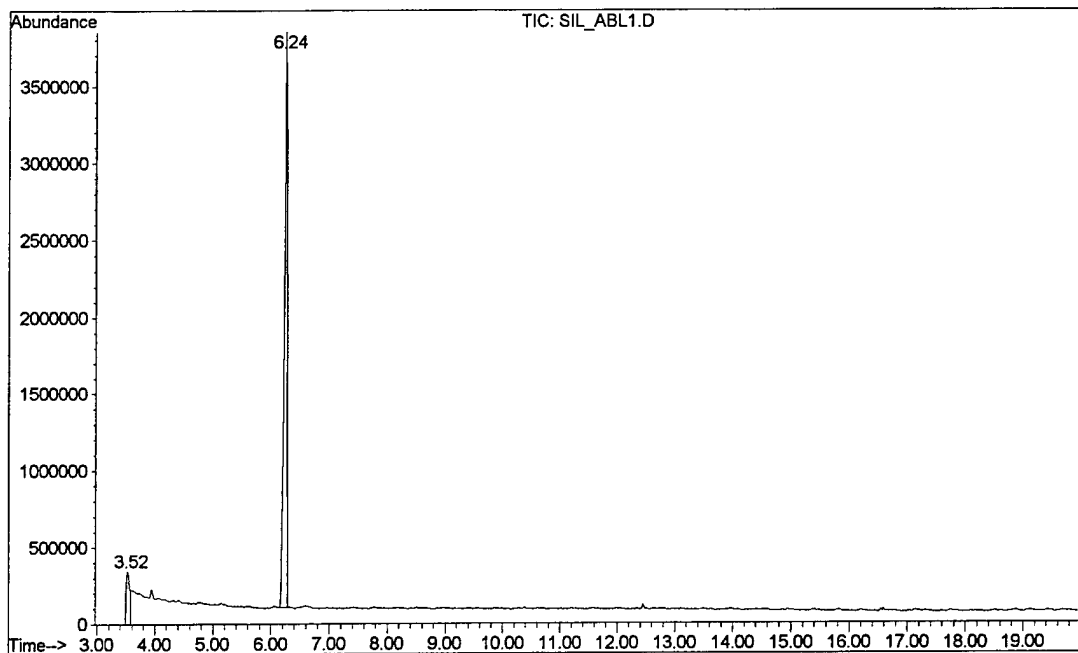
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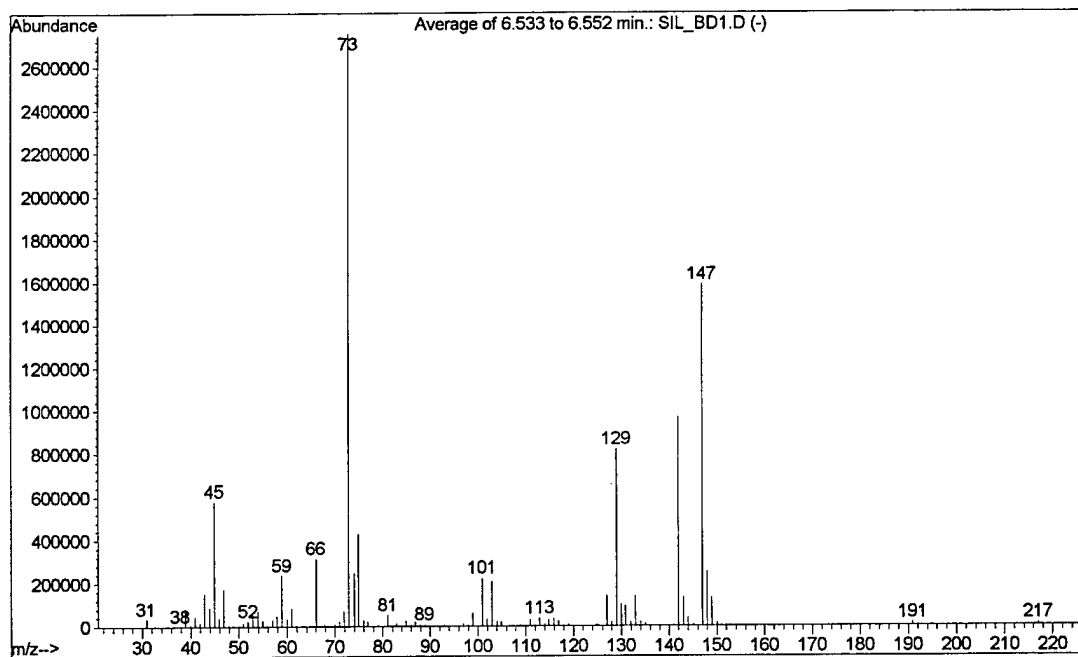
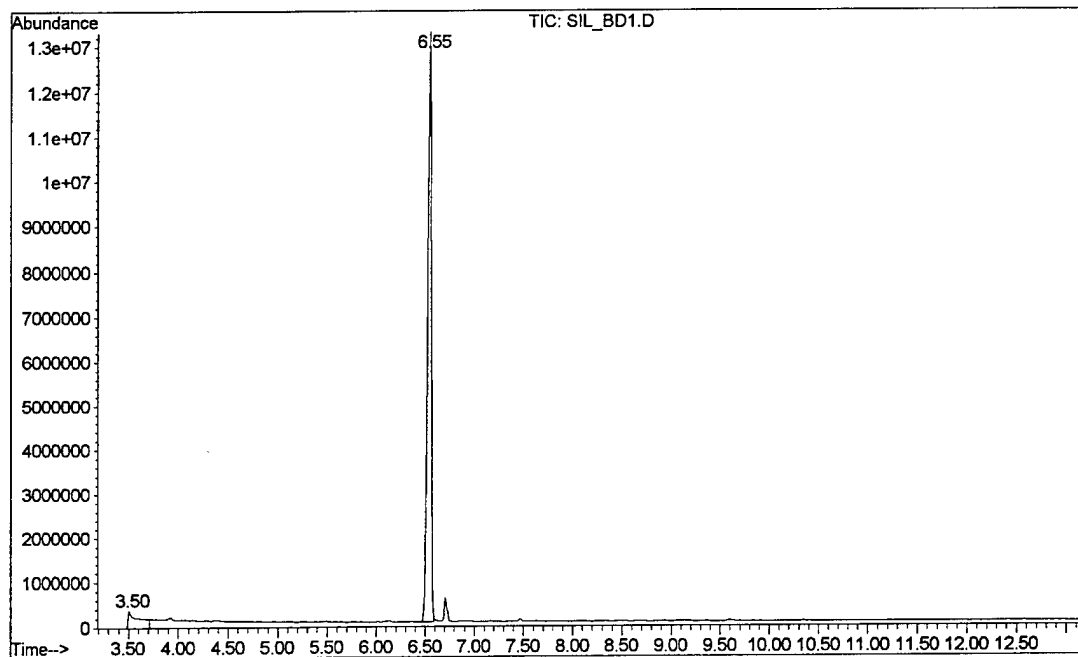
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Misc Info : ~40ppm each component From Silylation Reaction mix ERY
Vial Number: 0



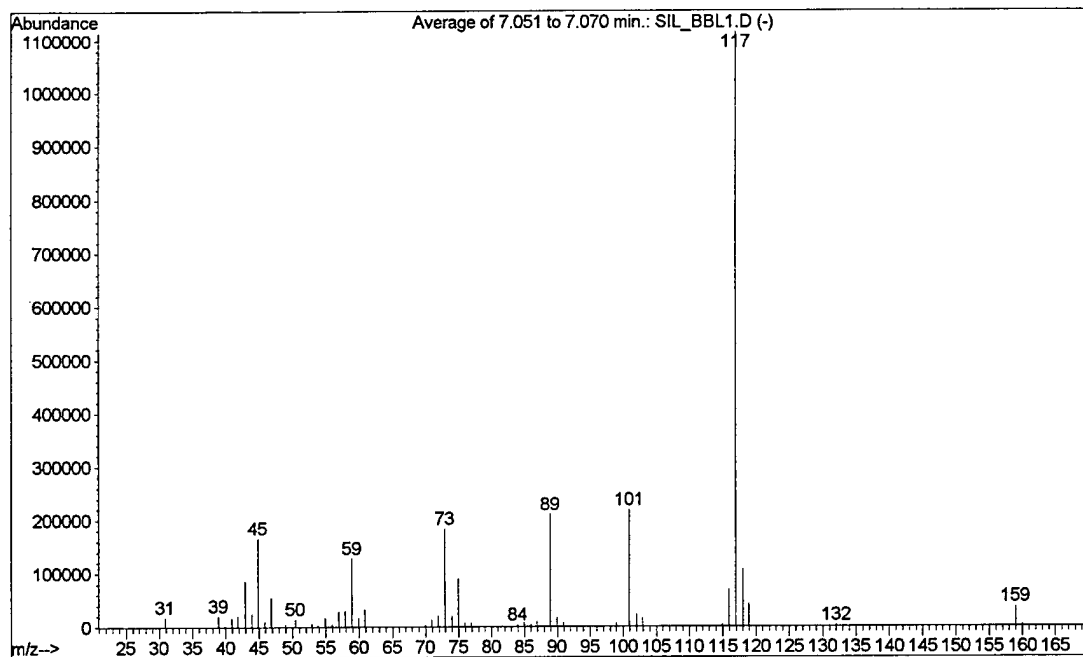
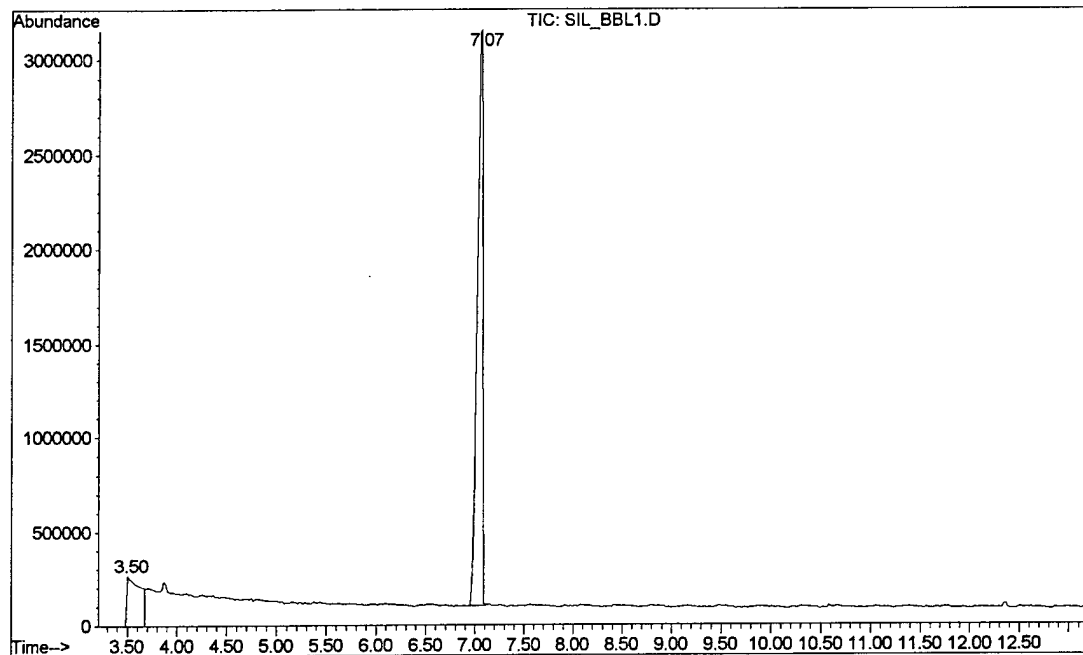
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Vial Number: 0



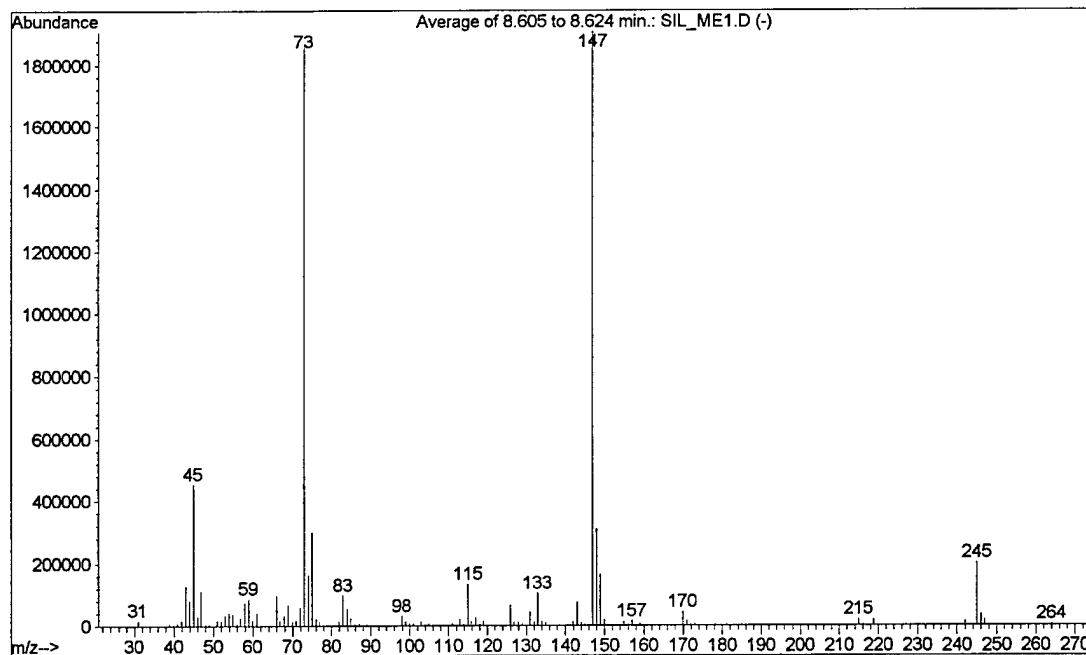
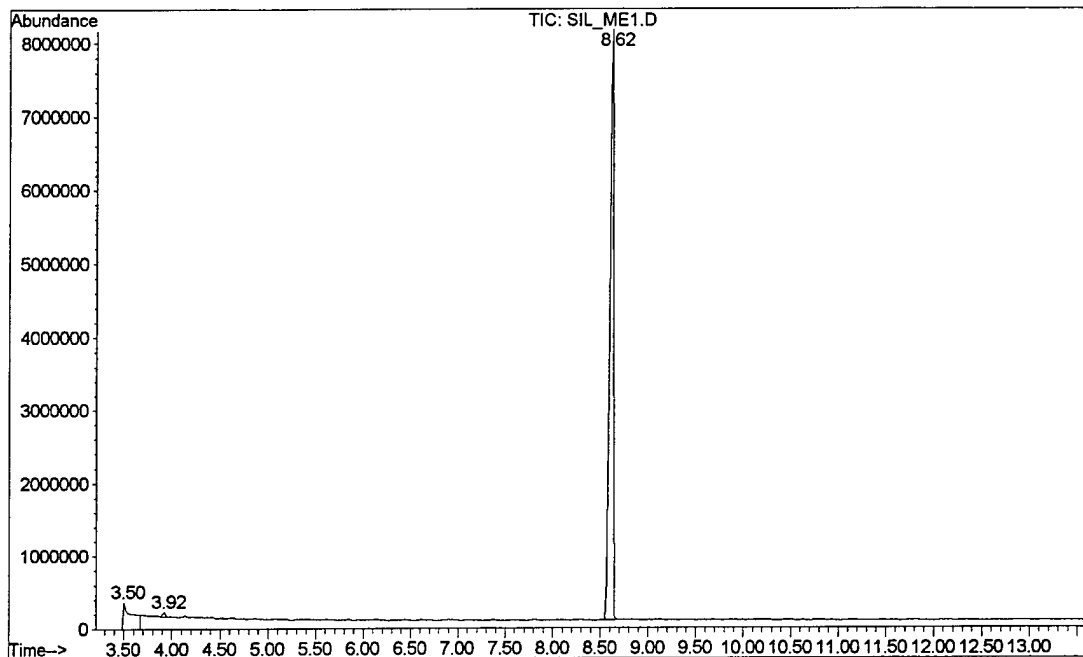
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Vial Number: 0



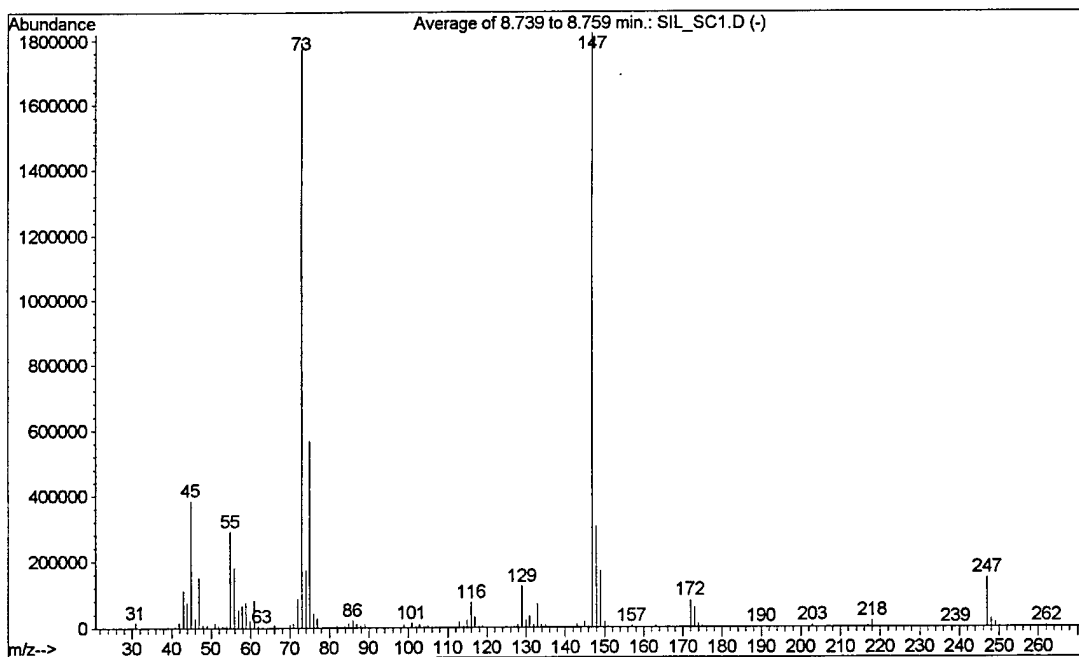
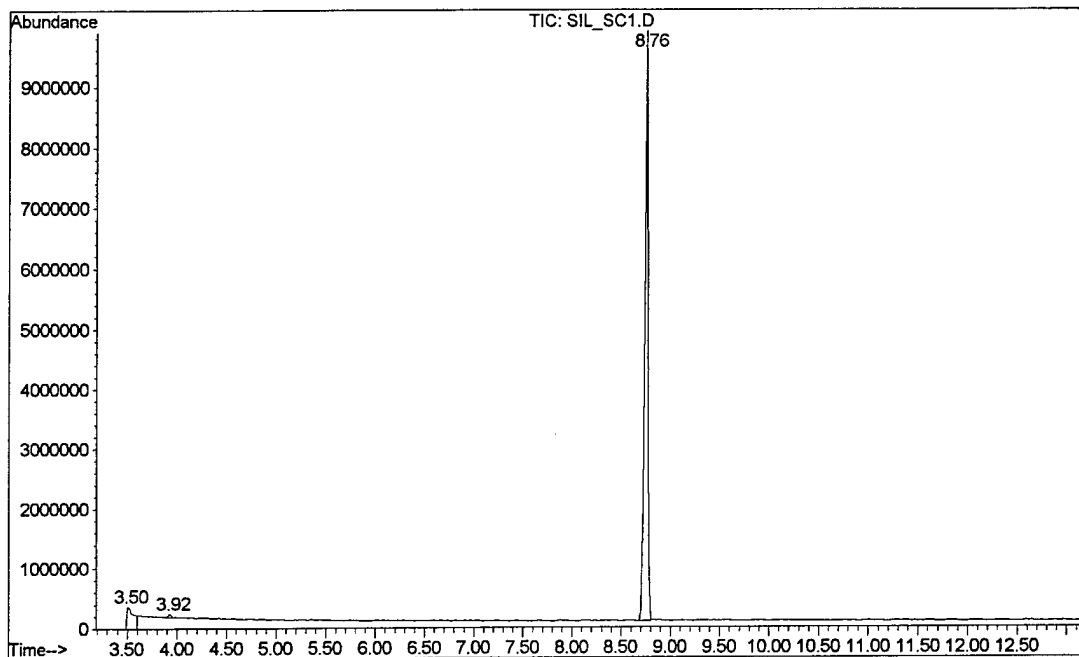
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Misc Info : 200ul beta-Hydroxy-Butyrolactone ref. solution + 200ul BSTFA
Vial Number: 0



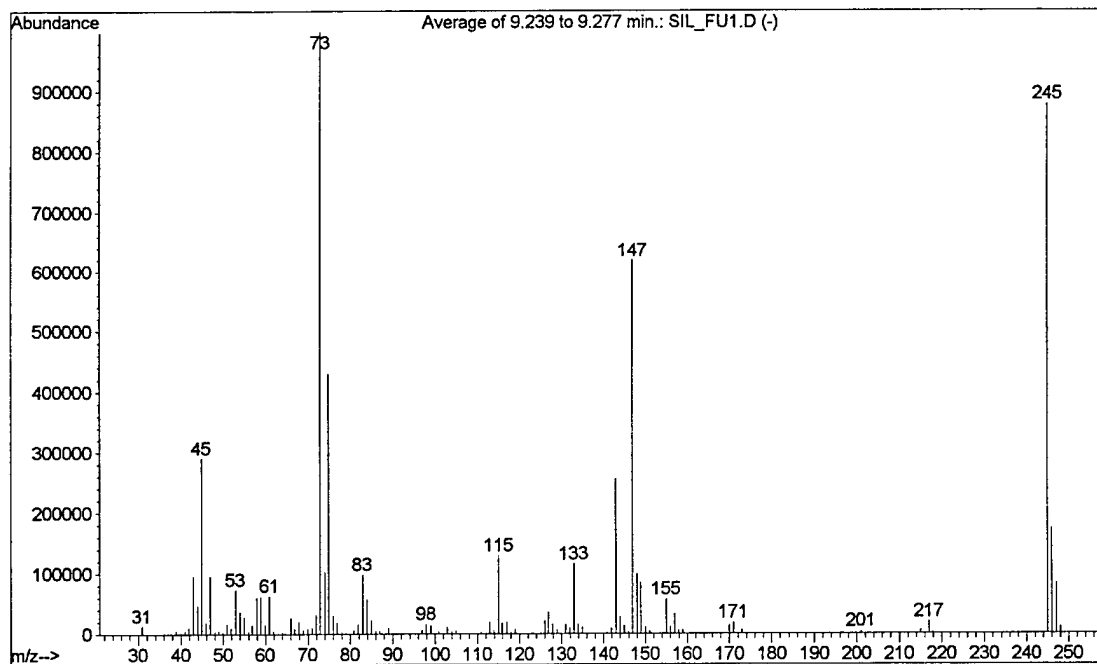
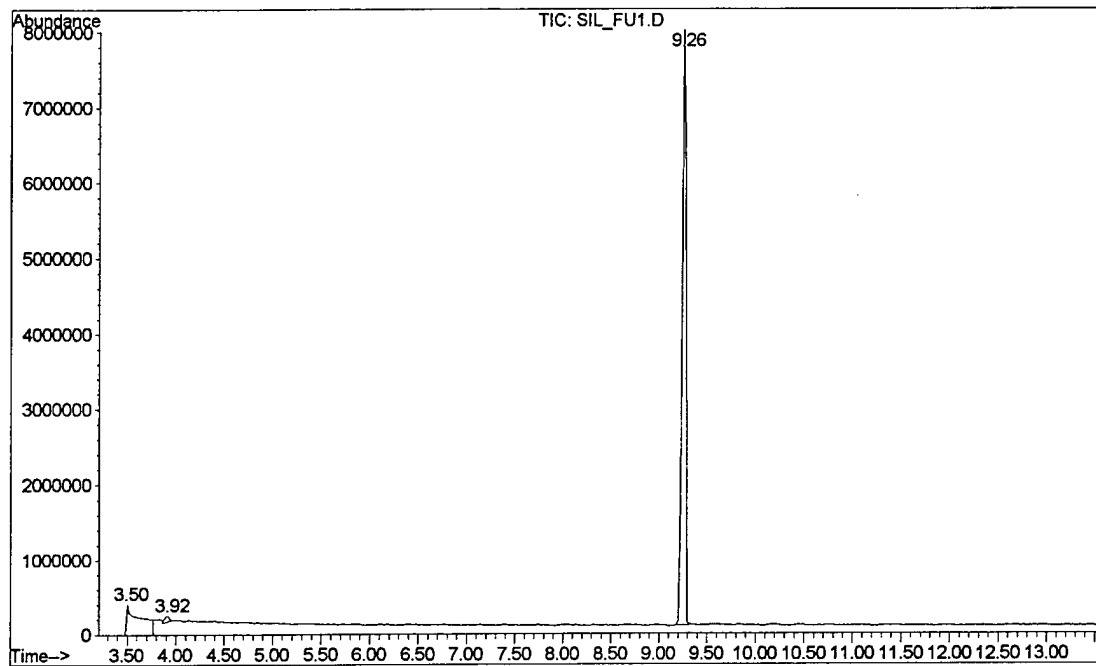
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Vial Number: 0



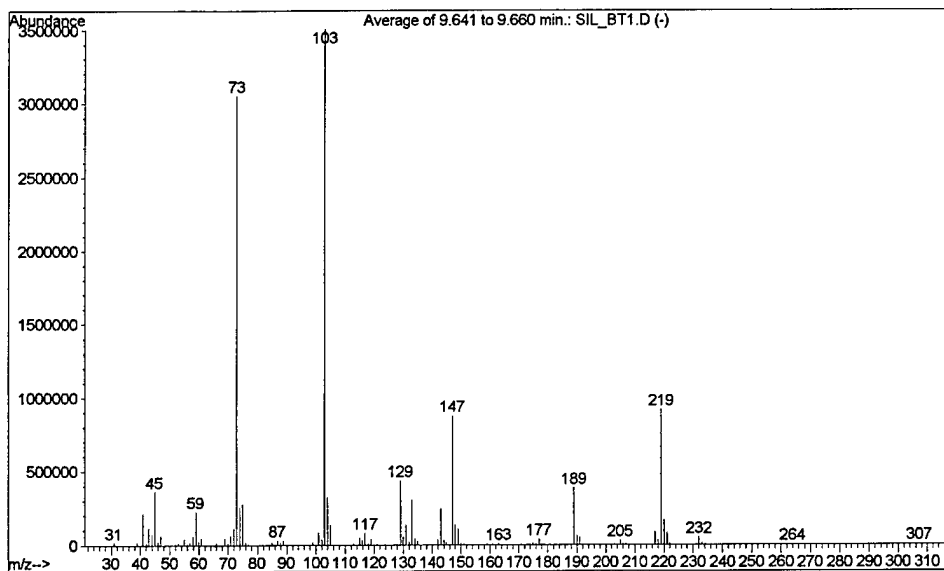
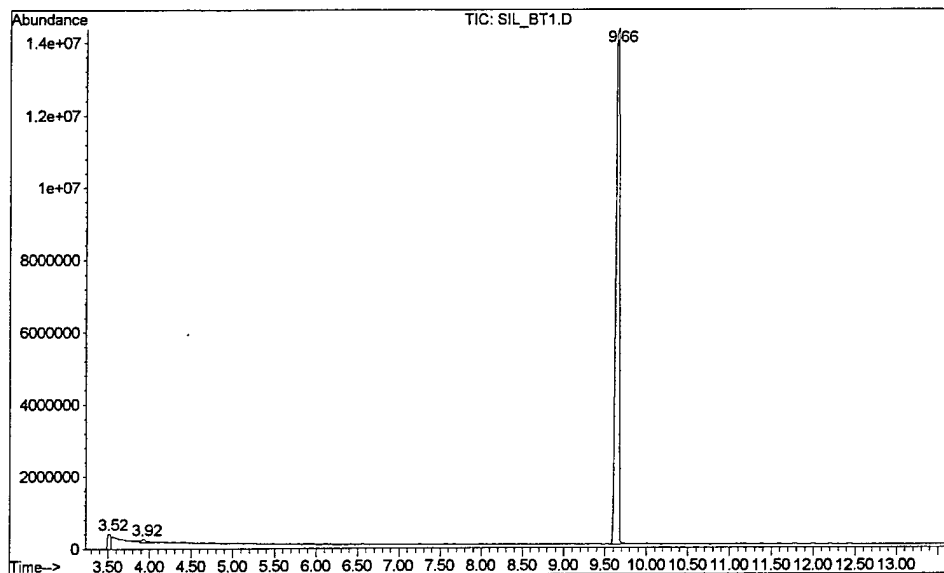
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Vial Number: 0



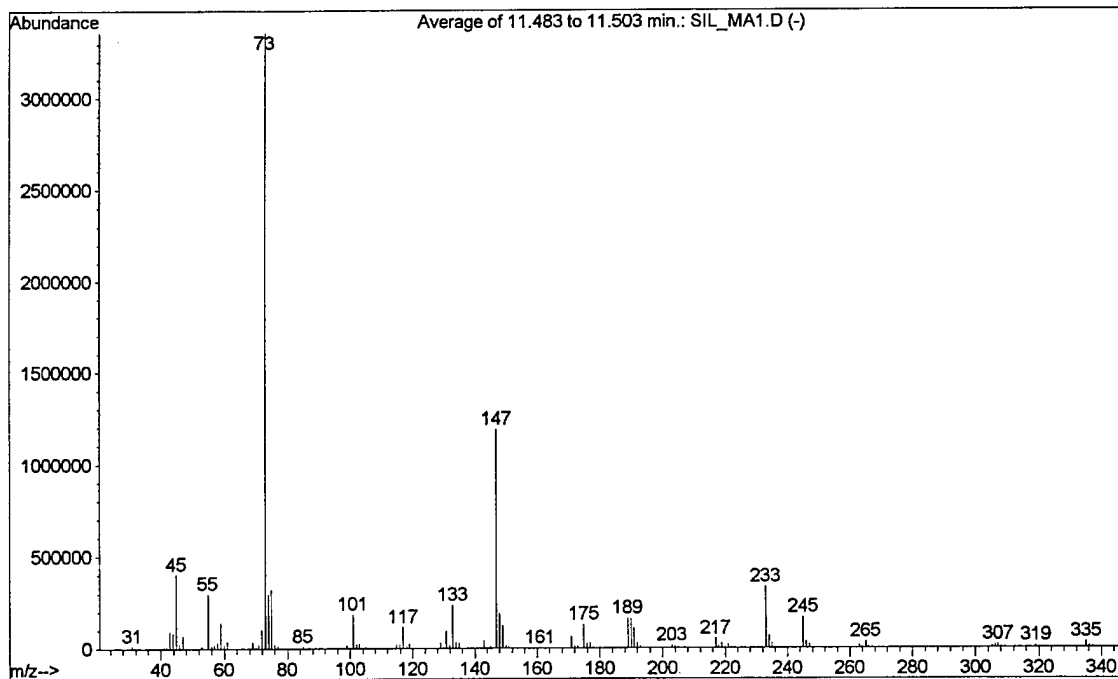
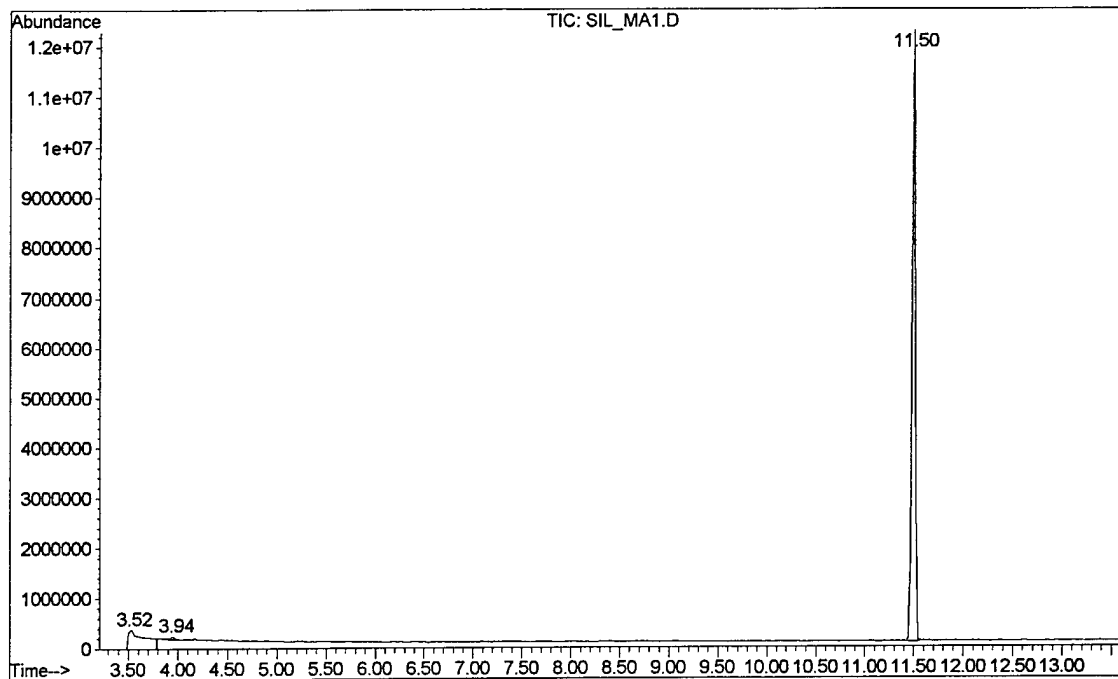
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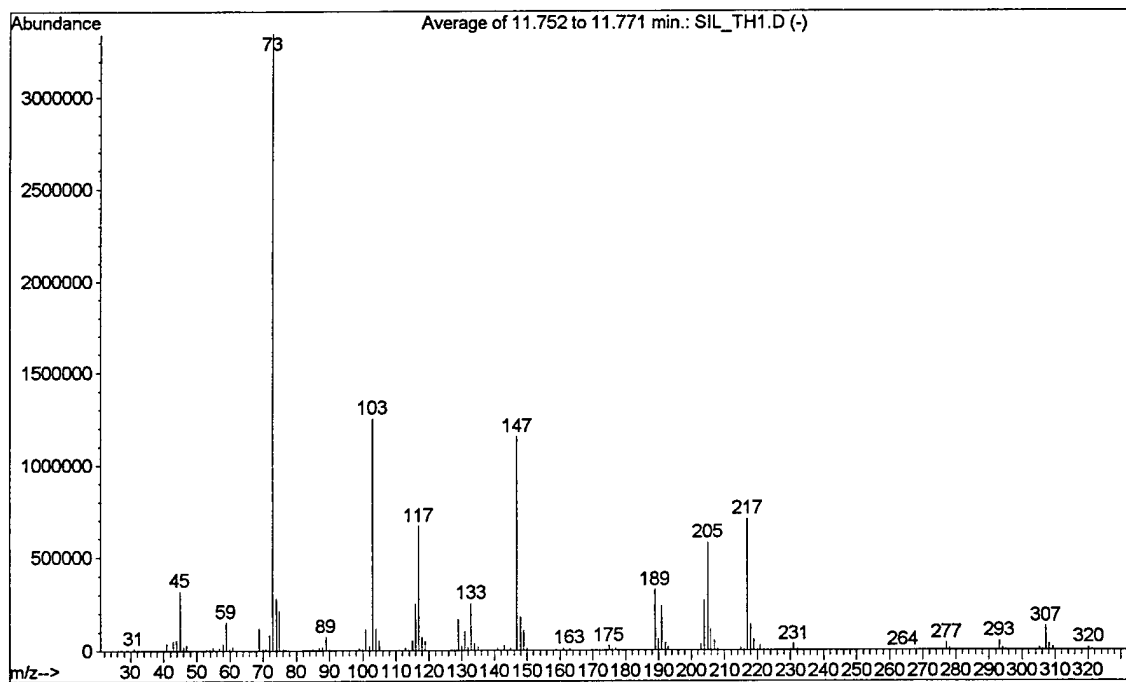
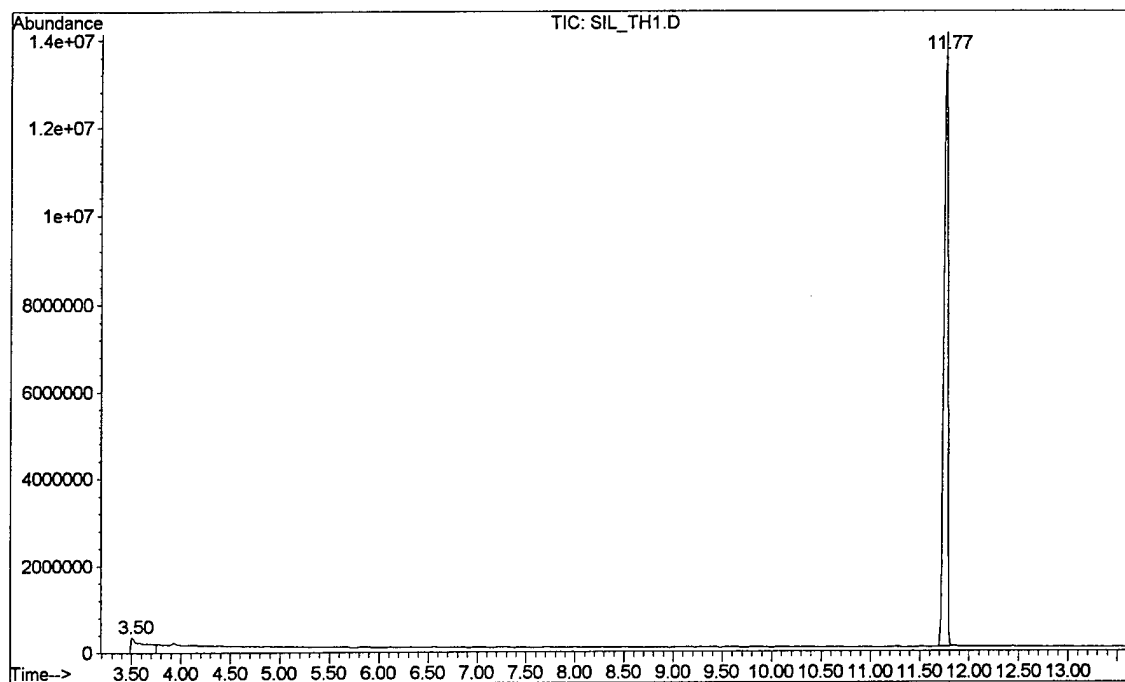
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Vial Number: 0



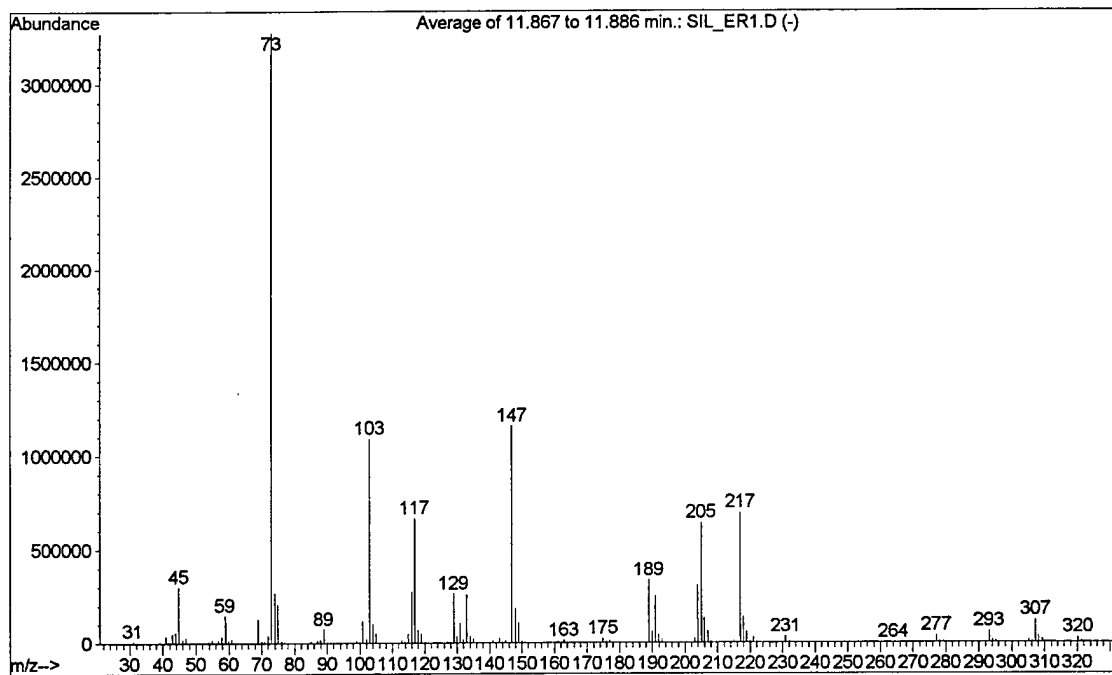
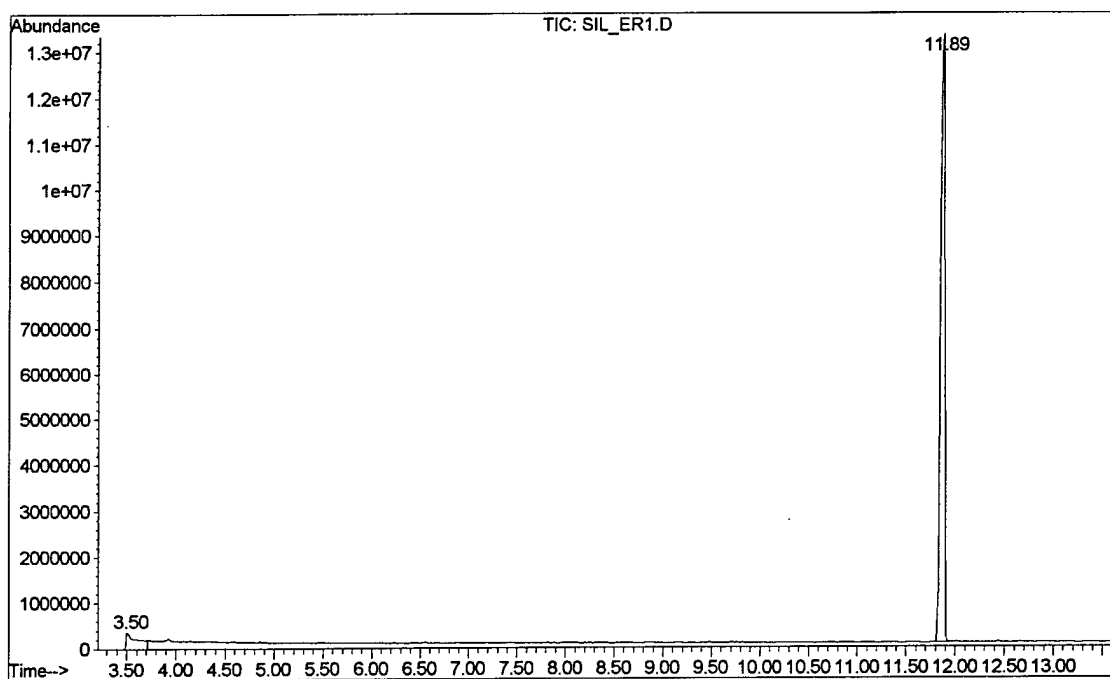
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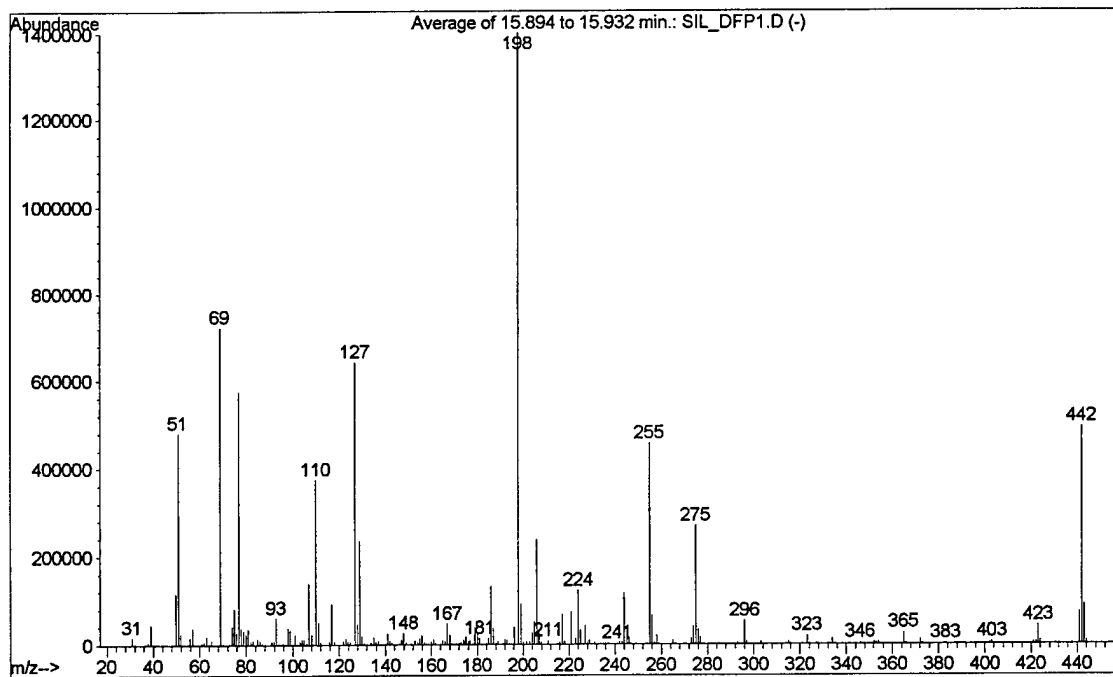
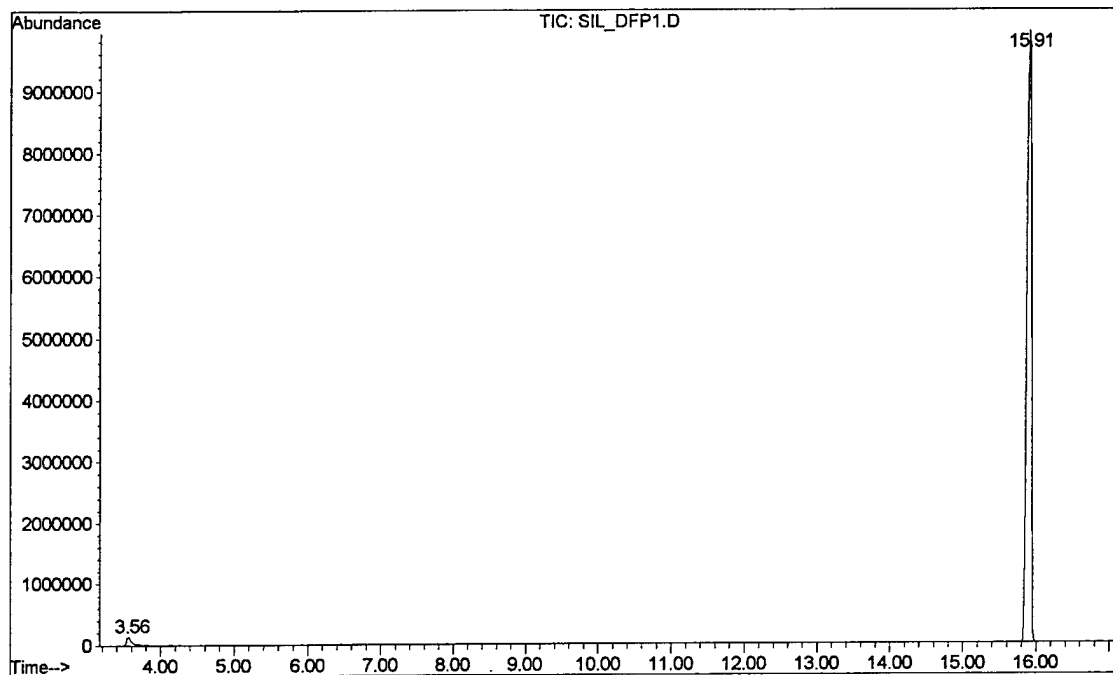
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Vial Number: 0



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Instrument : Finnigan
Sample Name: 400ppm_meso_Erythritol
Misc Info : 200ul meso_Erythritol ref. solution + 200ul BSTFA/1%TMCS +
Vial Number: 0



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Acquired : 29 Aug 2001 14:05 using AcqMethod METHOD.M
Instrument : Finnigan
Sample Name: 5000 ppm DFTPP
Misc Info : dftpp calibration for subsequent malic acid purity run 500
Vial Number: 0



Appendix B.
QC Data for Analytes

Table B-1. QC Data for AHL

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	8.25	0.0000	5.4010	2.8490	0
2	3.2700	0.0000	5.4010	-2.1310	0
3	3.3800	0.0000	5.4010	-2.0210	0
4	1.8900	0.0000	5.4010	-3.5110	0
5	18.4700	14.3000	12.9764	5.4936	1
6	11.6200	14.3000	12.9764	-1.3564	1
7	3.3800	14.3000	12.9764	-9.5964	1
8	1.8900	14.3000	12.9764	-11.0864	1
9	27.5100	28.6000	20.5518	6.9582	2
10	23.0400	28.6000	20.5518	2.4882	2
11	14.9400	28.6000	20.5518	-5.6118	2
12	25.3200	28.6000	20.5518	4.7682	2
13	38.8300	57.1000	35.6496	3.1804	3
14	51.5100	57.1000	35.6496	15.8604	3
15	23.3100	57.1000	35.6496	-12.3396	3
16	35.3200	57.1000	35.6496	-0.3296	3
17	134.0000	142.8000	81.0491	52.9509	4
18	82.2800	142.8000	81.0491	1.2309	4
19	74.6400	142.8000	81.0491	-6.4091	4
20	48.6600	142.8000	81.0491	-32.3891	4
21	246.2700	285.6000	156.6972	89.5728	4
22	175.3300	285.6000	156.6972	18.6328	5
23	75.4600	285.6000	156.6972	-81.2372	5
24	120.7300	285.6000	156.6972	-35.9672	5

Regression Output:

Constant	5.4010
Std Err of Y Est	30.8648
R Squared	0.7618
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.5297
Std Err of Coef.	0.0632

MDL = 140.16

Figure B-1

AHL Target versus Found

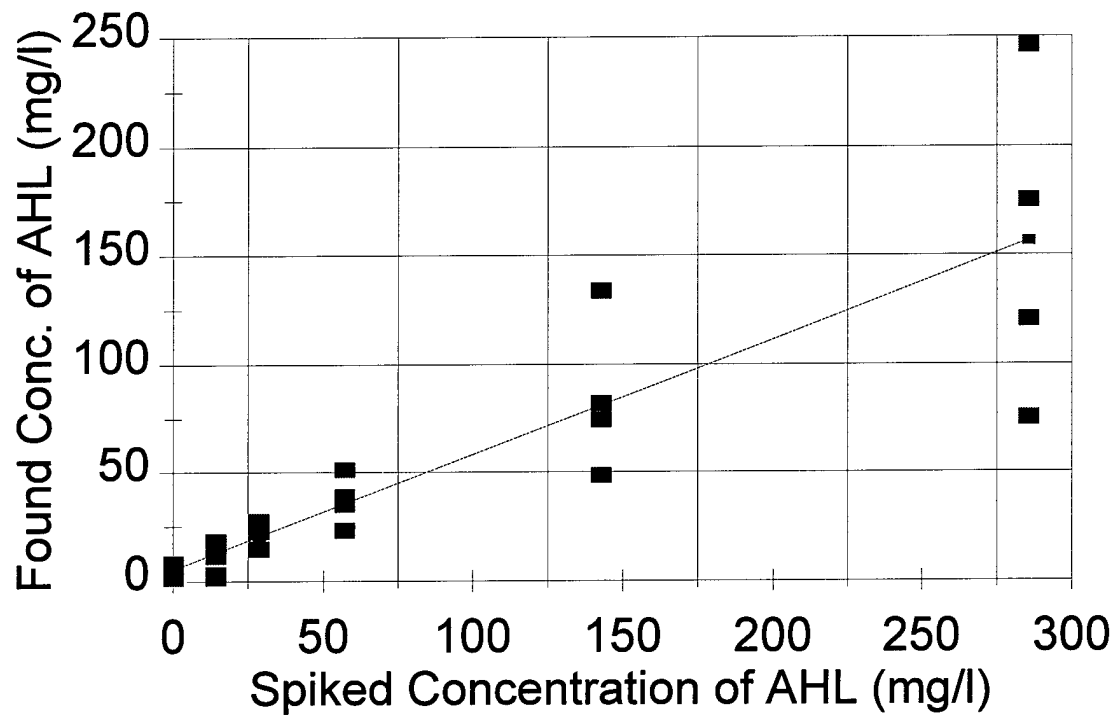


Table B-2. QC Data for BHL

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1	8.1000	0.0000	4.9523	3.1477	0
2	3.5100	0.0000	4.9523	-1.4423	0
3	3.5800	0.0000	4.9523	-1.3723	0
4	1.2900	0.0000	4.9523	-3.6623	0
5	18.1500	14.3000	16.6837	1.4663	1
6	16.5700	14.3000	16.6837	-0.1137	1
7	17.3700	14.3000	16.6837	0.6863	1
8	1.2900	14.3000	16.6837	-15.3937	1
9	27.6000	28.6000	28.4151	-0.8151	2
10	32.0100	28.6000	28.4151	3.5949	2
11	29.7000	28.6000	28.4151	1.2849	2
12	34.6500	28.6000	28.4151	6.2349	2
13	39.3700	57.2000	51.8780	-12.5080	3
14	74.5700	57.2000	51.8780	22.6920	3
15	57.0800	57.2000	51.8780	5.2020	3
16	42.3200	57.2000	51.8780	-9.5580	3
17	136.5000	143.1000	122.3486	14.1514	4
18	125.7200	143.1000	122.3486	3.3714	4
19	149.3100	143.1000	122.3486	26.9614	4
20	82.0300	143.1000	122.3486	-40.3186	4
21	270.7400	286.2000	239.7449	30.9951	5
22	278.6500	286.2000	239.7449	38.9051	5
23	172.4200	286.2000	239.7449	-67.3249	5
24	233.5600	286.2000	239.7449	-6.1849	5

Regression Output:

Constant	4.9523
Std Err of Y Est	22.0744
R Squared	0.9377
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.8204
Std Err of Coef.	0.0451

MDL= 100.24

Figure B-2.

Regression Line for BHL

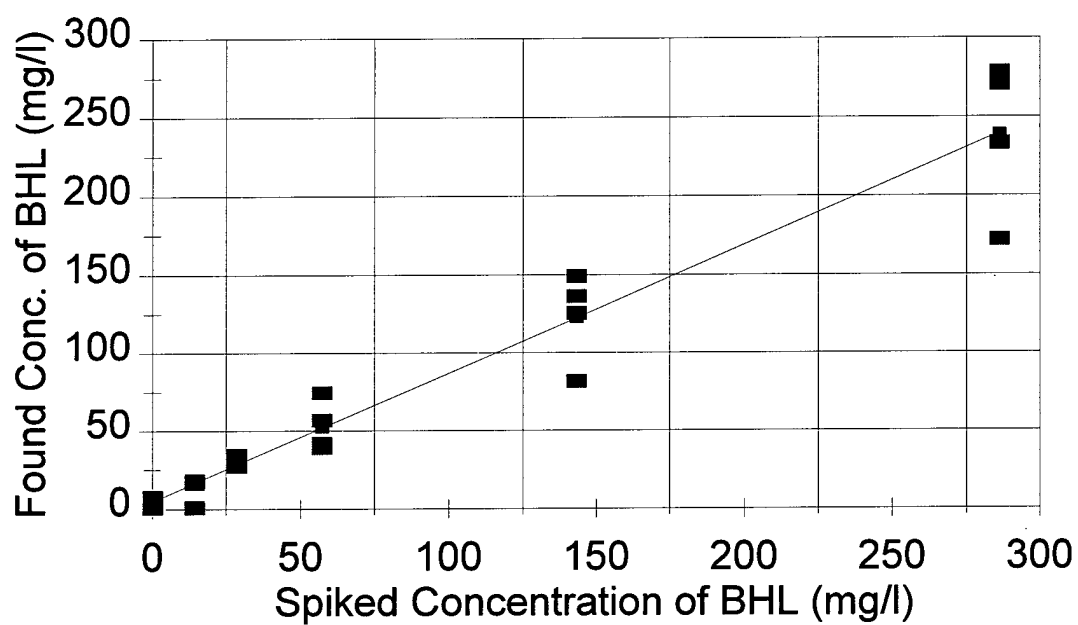


Table B-3. QC Data for BTB

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	15.5500	0.0000	6.2503	9.2997	0
2	6.4000	0.0000	6.2503	0.1497	0
3	7.3800	0.0000	6.2503	1.1297	0
4	3.0000	0.0000	6.2503	-3.2503	0
5	24.4200	18.5000	19.9607	4.4593	1
6	19.1200	18.5000	19.9607	-0.8407	1
7	25.0900	18.5000	19.9607	5.1293	1
8	3.0000	18.5000	19.9607	-16.9607	1
9	38.8300	37.1000	33.7452	5.0848	2
10	43.2600	37.1000	33.7452	9.5148	2
11	37.6300	37.1000	33.7452	3.8848	2
12	16.9700	37.1000	33.7452	-16.7752	2
13	36.6000	74.1000	61.1661	-24.5661	3
14	94.0300	74.1000	61.1661	32.8639	3
15	73.1500	74.1000	61.1661	11.9839	3
16	20.9600	74.1000	61.1661	-40.2061	3
17	167.7500	185.3000	143.5768	24.1732	4
18	157.3600	185.3000	143.5768	13.7832	4
19	188.7700	185.3000	143.5768	45.1932	4
20	90.1800	185.3000	143.5768	-53.3968	4
21	294.9300	370.6000	280.9034	14.0266	5
22	332.1500	370.6000	280.9034	51.2466	5
23	205.0100	370.6000	280.9034	-75.8934	5
24	280.8700	370.6000	280.9034	-0.0334	5

Regression Output:

Constant	6.2503
Std Err of Y Est	29.0299
R Squared	0.9226
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.7411
Std Err of Coef.	0.0458

MDL= 131.82

Figure B-3.

Regression Line for BTB

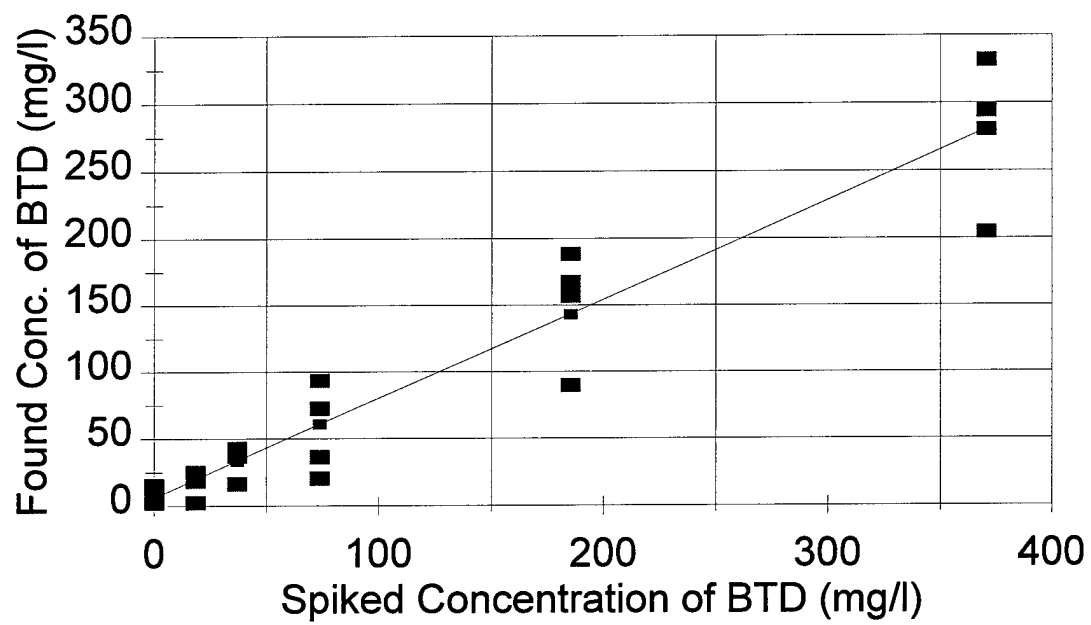


Table B-4. QC Data for MLE

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	7.4100	0.0000	-2.7673	10.1773	0
2	8.9000	0.0000	-2.7673	11.6673	0
3	18.2900	0.0000	-2.7673	21.0573	0
4	0.4100	0.0000	-2.7673	3.1773	0
5	7.4100	18.0000	5.2660	2.1440	1
6	8.9000	18.0000	5.2660	3.6340	1
7	18.2900	18.0000	5.2660	13.0240	1
8	0.4100	18.0000	5.2660	-4.8560	1
9	15.82	36.0000	13.2993	2.5207	2
10	8.9	36.0000	13.2993	-4.3993	2
11	18.29	36.0000	13.2993	4.9907	2
12	0.41	36.0000	13.2993	-12.8893	2
13	16.5100	72.1000	29.4104	-12.9004	3
14	27.1100	72.1000	29.4104	-2.3004	3
15	40.6500	72.1000	29.4104	11.2396	3
16	0.4100	72.1000	29.4104	-29.0004	3
17	66.3400	180.2000	77.6547	-11.3147	4
18	68.5000	180.2000	77.6547	-9.1547	4
19	90.77	180.2000	77.6547	13.1153	4
20	36.6700	180.2000	77.6547	-40.9847	4
21	232.8600	360.3000	158.0320	74.8280	5
22	181.3700	360.3000	158.0320	23.3380	5
23	125.9400	360.3000	158.0320	-32.0920	5
24	123.0100	360.3000	158.0320	-35.0220	5

Regression Output:

Constant	-2.7673
Std Err of Y Est	24.0491
R Squared	0.8561
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.4463
Std Err of Coef.	0.0390

MDL= 109.21

Figure B-4.

Regression Line for MLE

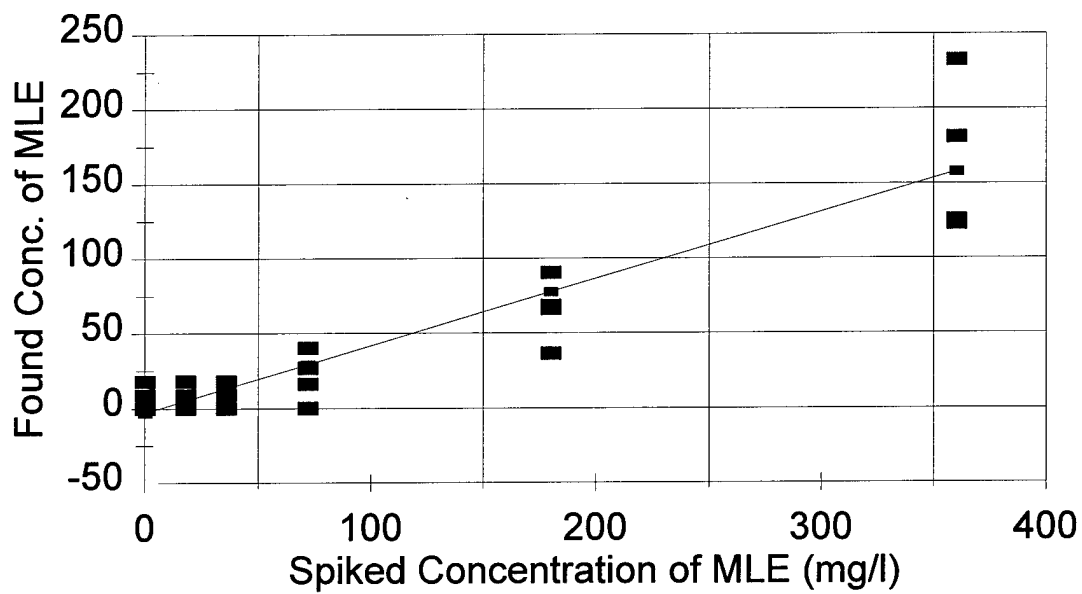


Table B-5. QC Data for SUC

	C(found) Y	TC X	C(calc)	Delta	Group
1	14.0500	0.0000	1.9761	12.0739	0
2	6.2800	0.0000	1.9761	4.3039	0
3	7.4600	0.0000	1.9761	5.4839	0
4	-10.7500	0.0000	1.9761	-12.7261	0
5	24.4000	17.9000	18.2501	6.1499	1
6	21.0100	17.9000	18.2501	2.7599	1
7	21.0600	17.9000	18.2501	2.8099	1
8	4.2300	17.9000	18.2501	-14.0201	1
9	40.4700	35.9000	34.6151	5.8549	2
10	40.0600	35.9000	34.6151	5.4449	2
11	35.8300	35.9000	34.6151	1.2149	2
12	30.8000	35.9000	34.6151	-3.8151	2
13	54.5300	71.7000	67.1632	-12.6332	3
14	89.7800	71.7000	67.1632	22.6168	3
15	64.0800	71.7000	67.1632	-3.0832	3
16	42.1000	71.7000	67.1632	-25.0632	3
17	176.0200	179.3000	164.9892	11.0308	4
18	185.3200	179.3000	164.9892	20.3308	4
19	187.6700	179.3000	164.9892	22.6808	4
20	110.4500	179.3000	164.9892	-54.5392	4
21	384.5000	358.5000	327.9113	56.5887	5
22	399.3800	358.5000	327.9113	71.4687	5
23	213.6900	358.5000	327.9113	-114.2213	5
24	317.2000	358.5000	327.9113	-10.7113	5

Regression Output:

Constant	1.9761
Std Err of Y Est	35.3491
R Squared	0.9188
No. of Observations	24.0000
Degrees of Freedom	22.0000

X Coefficient(s)	0.9092
Std Err of Coef.	0.0576

MDL= 160.52

Figure B-5.

Regression Line for SUC

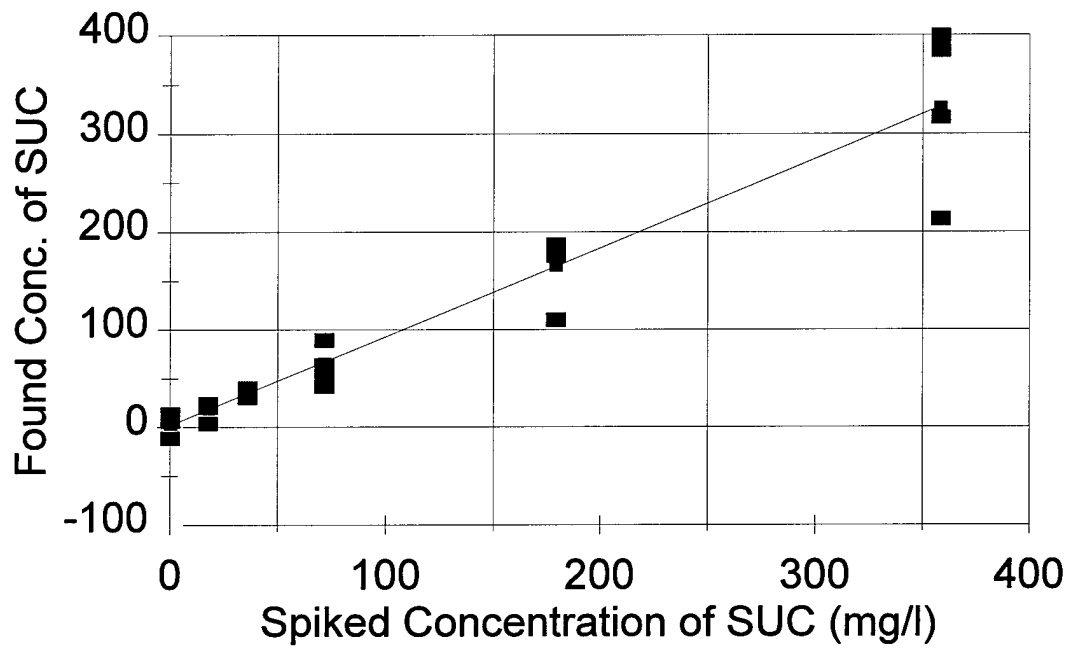


Table B-6. QC Data for FUM

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	11.0600	0.0000	-11.2095	22.2695	0
2	0.3300	0.0000	-11.2095	11.5395	0
3	9.7600	0.0000	-11.2095	20.9695	0
4	-0.5900	0.0000	-11.2095	10.6195	0
5	11.0600	18.0000	0.2436	10.8164	1
6	0.3300	18.0000	0.2436	0.0864	1
7	9.7600	18.0000	0.2436	9.5164	1
8	-0.5900	18.0000	0.2436	-0.8336	1
9	11.0600	36.0000	11.6967	-0.6367	2
10	0.3300	36.0000	11.6967	-11.3667	2
11	9.7600	36.0000	11.6967	-1.9367	2
12	22.1200	36.0000	11.6967	10.4233	2
13	21.0700	72.1000	34.6666	-13.5966	3
14	15.5700	72.1000	34.6666	-19.0966	3
15	23.9700	72.1000	34.6666	-10.6966	3
16	24.7300	72.1000	34.6666	-9.9366	3
17	89.2000	180.2000	103.4489	-14.2489	4
18	71.3700	180.2000	103.4489	-32.0789	4
19	96.7200	180.2000	103.4489	-6.7289	4
20	80.1000	180.2000	103.4489	-23.3489	4
21	283.2100	360.3000	218.0436	65.1664	5
22	243.2700	360.3000	218.0436	25.2264	5
23	143.9700	360.3000	218.0436	-74.0736	5
24	249.9800	360.3000	218.0436	31.9364	5

Regression Output:

Constant	-11.2095
Std Err of Y Est	26.6344
R Squared	0.9079
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.6363
Std Err of Coef.	0.0432

MDL= 120.95

Figure B-6.

Regression Line for FUM

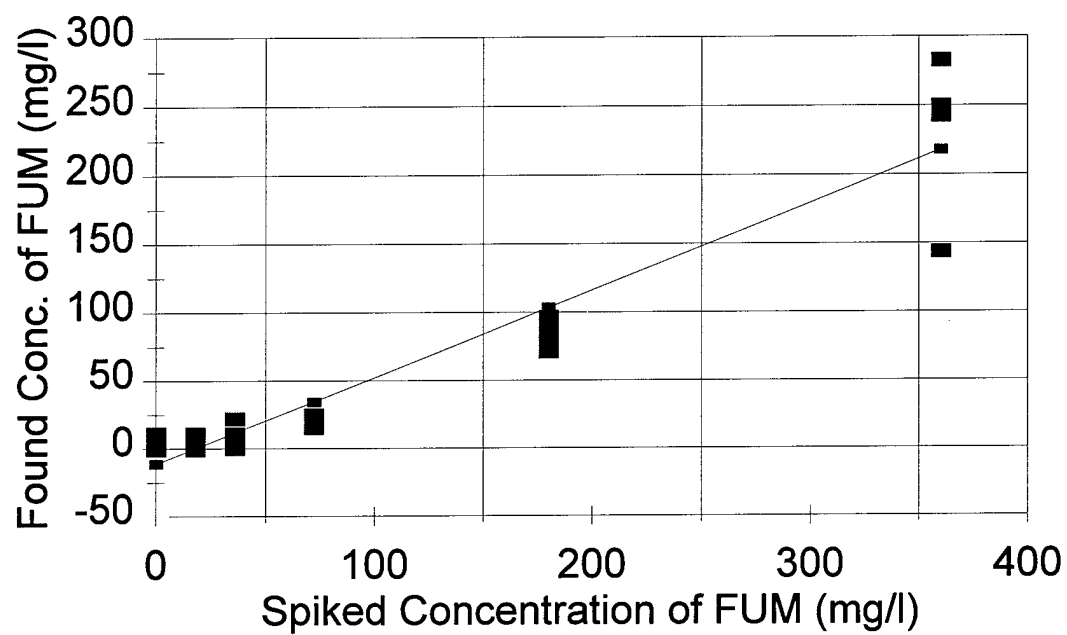


Table B-7. QC Data for MAL

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	21.9500	0.0000	7.2045	14.7455	0
2	26.9500	0.0000	7.2045	19.7455	0
3	22.1100	0.0000	7.2045	14.9055	0
4	5.2700	0.0000	7.2045	-1.9345	0
5	31.0100	24.5000	26.4651	4.5449	1
6	27.2600	24.5000	26.4651	0.7949	1
7	37.2400	24.5000	26.4651	10.7749	1
8	24.3200	24.5000	26.4651	-2.1451	1
9	36.7800	48.9000	45.6472	-8.8672	2
10	38.1900	48.9000	45.6472	-7.4572	2
11	58.7100	48.9000	45.6472	13.0628	2
12	35.2200	48.9000	45.6472	-10.4272	2
13	73.8400	97.8000	84.0899	-10.2499	3
14	67.1700	97.8000	84.0899	-16.9199	3
15	92.8100	97.8000	84.0899	8.7201	3
16	51.4600	97.8000	84.0899	-32.6299	3
17	213.2500	244.6000	199.4967	13.7533	4
18	167.6300	244.6000	199.4967	-31.8667	4
19	270.9400	244.6000	199.4967	71.4433	4
20	131.0800	244.6000	199.4967	-68.4167	4
21	469.5500	489.2000	391.7890	77.7610	5
22	460.5200	489.2000	391.7890	68.7310	5
23	281.8400	489.2000	391.7890	-109.9490	5
24	373.6700	489.2000	391.7890	-18.1190	5

Regression Output:

Constant	7.2045
Std Err of Y Est	41.0644
R Squared	0.9211
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.7861
Std Err of Coef.	0.0491

MDL= 186.47

Figure B-7.

Regression Line for MAL

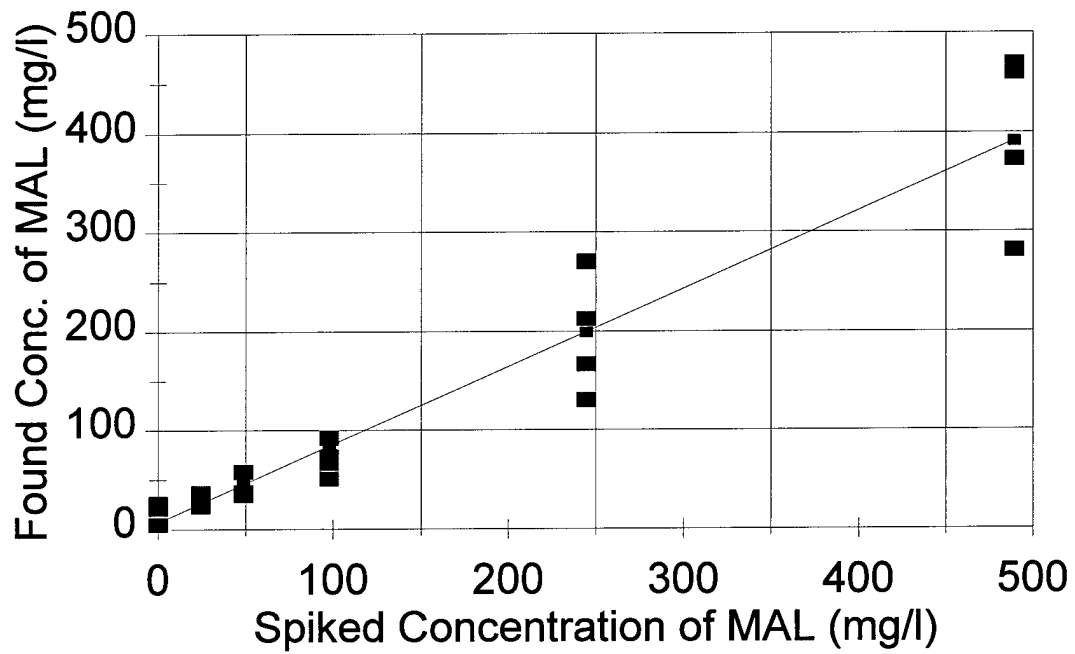


Table B-8. QC Data for BUT

	C(found) Y	TC X	C(calc)	Delta	Group
1	21.1500	0.0000	12.4835	8.6665	0
2	11.6200	0.0000	12.4835	-0.8635	0
3	11.9800	0.0000	12.4835	-0.5035	0
4	5.2700	0.0000	12.4835	-7.2135	0
5	36.5000	24.9000	37.1552	-0.6552	1
6	30.2100	24.9000	37.1552	-6.9452	1
7	42.9400	24.9000	37.1552	5.7848	1
8	24.1700	24.9000	37.1552	-12.9852	1
9	54.1500	49.8000	61.8269	-7.6769	2
10	70.5500	49.8000	61.8269	8.7231	2
11	76.4200	49.8000	61.8269	14.5931	2
12	59.5500	49.8000	61.8269	-2.2769	2
13	58.1200	99.5000	111.0713	-52.9513	3
14	168.3100	99.5000	111.0713	57.2387	3
15	139.1200	99.5000	111.0713	28.0487	3
16	78.0300	99.5000	111.0713	-33.0413	3
17	230.8900	248.8000	259.0025	-28.1125	4
18	288.9000	248.8000	259.0025	29.8975	4
19	345.9200	248.8000	259.0025	86.9175	4
20	175.9700	248.8000	259.0025	-83.0325	4
21	453.2500	453.2500	461.5782	-8.3282	5
22	579.4500	453.2500	461.5782	117.8718	5
23	345.3900	453.2500	461.5782	-116.1882	5
24	464.6100	453.2500	461.5782	3.0318	5

Regression Output:

Constant	12.4835
Std Err of Y Est	48.7554
R Squared	0.9196
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.9908
Std Err of Coef.	0.0625

MDL= 221.40

Figure 8.

Regression Line for BUT

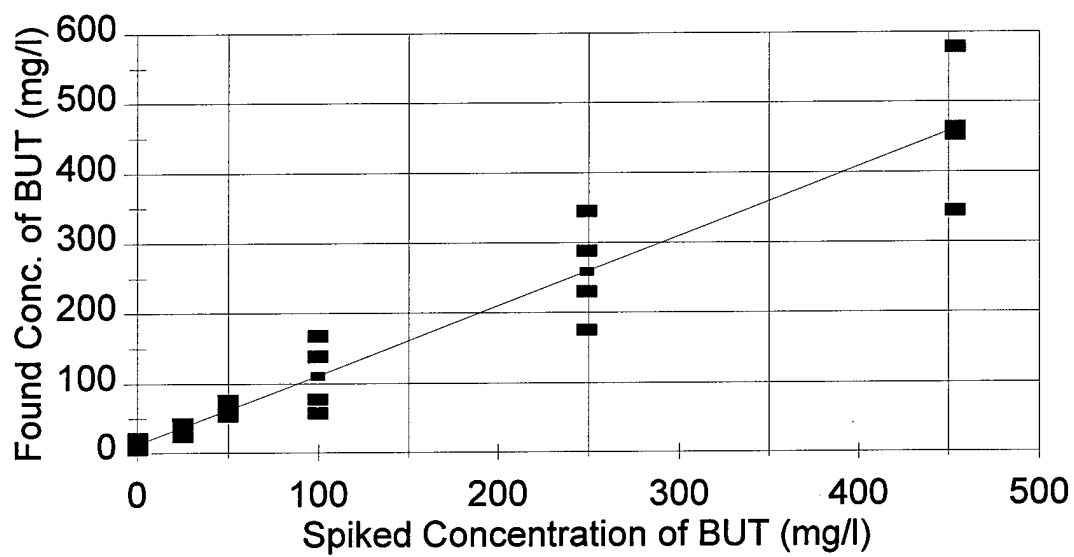


Table B-9. QC Data for ERY

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	9.6600	0.0000	8.6035	1.0565	0
2	5.1200	0.0000	8.6035	-3.4835	0
3	5.0800	0.0000	8.6035	-3.5235	0
4	4.9700	0.0000	8.6035	-3.6335	0
5	15.5000	12.2000	19.7923	-4.2923	1
6	11.4100	12.2000	19.7923	-8.3823	1
7	19.9200	12.2000	19.7923	0.1277	1
8	14.1700	12.2000	19.7923	-5.6223	1
9	24.4400	24.3000	30.8894	-6.4494	2
10	31.0700	24.3000	30.8894	0.1806	2
11	37.8000	24.3000	30.8894	6.9106	2
12	33.2800	24.3000	30.8894	2.3906	2
13	34.6200	48.7000	53.2669	-18.6469	3
14	78.0200	48.7000	53.2669	24.7531	3
15	69.5900	48.7000	53.2669	16.3231	3
16	40.9100	48.7000	53.2669	-12.3569	3
17	121.3500	121.7000	120.2161	1.1339	4
18	136.5800	121.7000	120.2161	16.3639	4
19	168.8800	121.7000	120.2161	48.6639	4
20	86.1800	121.7000	120.2161	-34.0361	4
21	251.2000	243.3000	231.7369	19.4631	5
22	277.5900	243.3000	231.7369	45.8531	5
23	165.4000	243.3000	231.7369	-66.3369	5
24	215.2800	243.3000	231.7369	-16.4569	5

Regression Output:

Constant	8.60353731
	77511
Std Err of Y Est	23.9148773
	081627
R Squared	0.92055824
	1901118
No. of Observations	24
Degrees of Freedom	22
X Coefficient(s)	0.91711189
	7142366
Std Err of Coef.	0.05743938
	41662897

MDL= 108.60

Figure B-9.

Regression Line for ERY

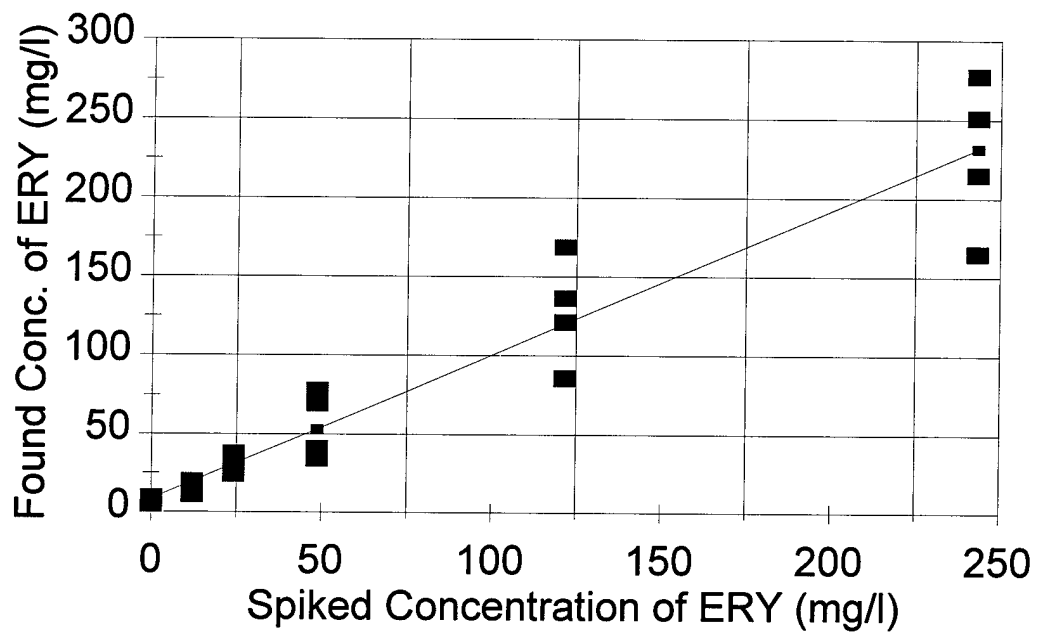


Table B-10. QC Data for THR

	C(found) Y	C(Target) X	C(calc)	Delta	Group
1	9.4700	0.0000	6.9359	2.5341	0
2	4.9500	0.0000	6.9359	-1.9859	0
3	4.9900	0.0000	6.9359	-1.9459	0
4	2.5000	0.0000	6.9359	-4.4359	0
5	14.5500	12.0000	18.2198	-3.6698	1
6	9.8800	12.0000	18.2198	-8.3398	1
7	19.0000	12.0000	18.2198	0.7802	1
8	11.3500	12.0000	18.2198	-6.8698	1
9	21.9600	24.0000	29.5038	-7.5438	2
10	26.9500	24.0000	29.5038	-2.5538	2
11	36.8600	24.0000	29.5038	7.3562	2
12	32.0800	24.0000	29.5038	2.5762	2
13	34.7700	48.1000	52.1656	-17.3956	3
14	74.2400	48.1000	52.1656	22.0744	3
15	67.1100	48.1000	52.1656	14.9444	3
16	43.3700	48.1000	52.1656	-8.7956	3
17	121.3600	120.2000	119.9633	1.3967	4
18	135.7100	120.2000	119.9633	15.7467	4
19	163.0000	120.2000	119.9633	43.0367	4
20	88.8300	120.2000	119.9633	-31.1333	4
21	252.3400	240.3000	232.8966	19.4434	5
22	278.1800	240.3000	232.8966	45.2834	5
23	159.5200	240.3000	232.8966	-73.3766	5
24	225.7700	240.3000	232.8966	-7.1266	5

Regression Output:

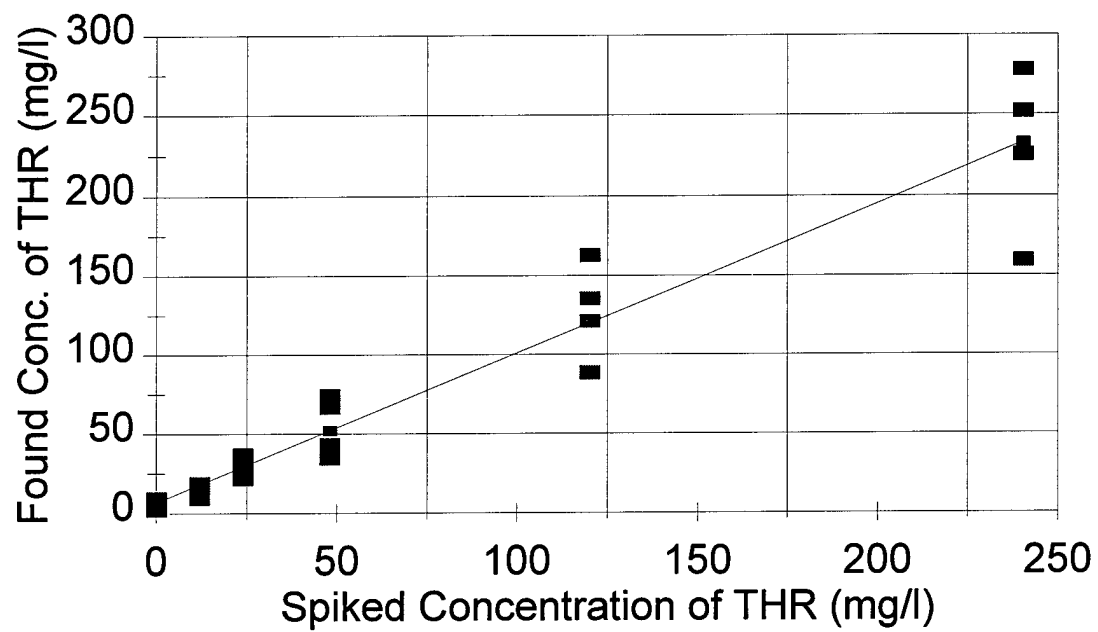
Constant	6.9359
Std Err of Y Est	23.6589
R Squared	0.9239
No. of Observations	24
Degrees of Freedom	22

X Coefficient(s)	0.9403
Std Err of Coef.	0.0575

MDL= 107.43

Figure B-10.

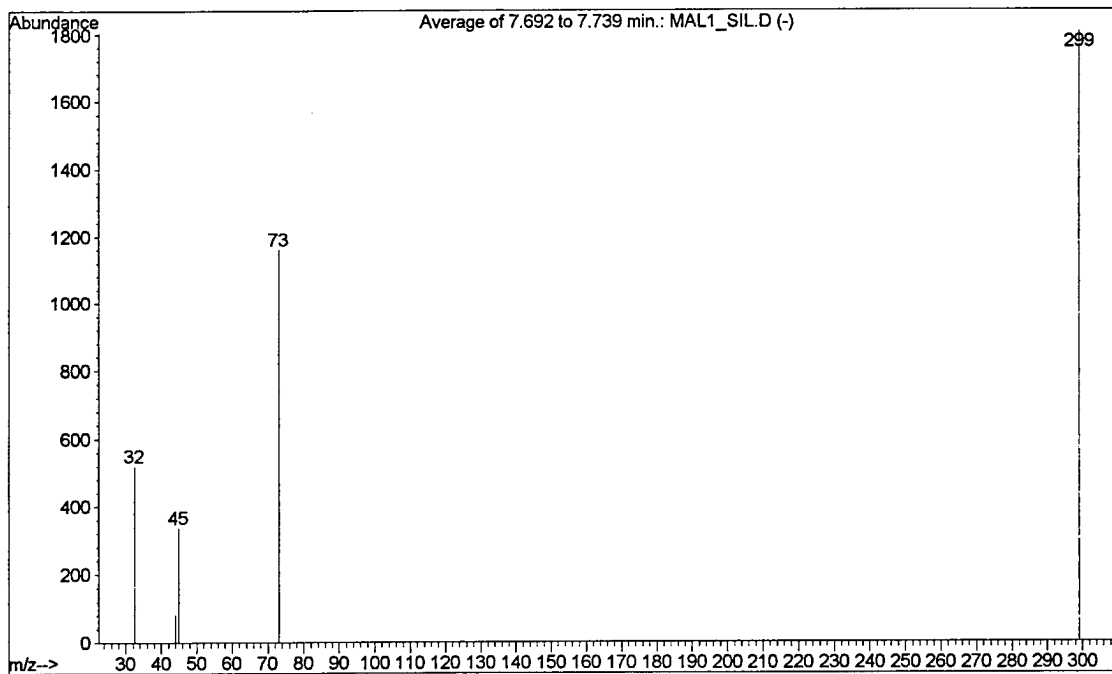
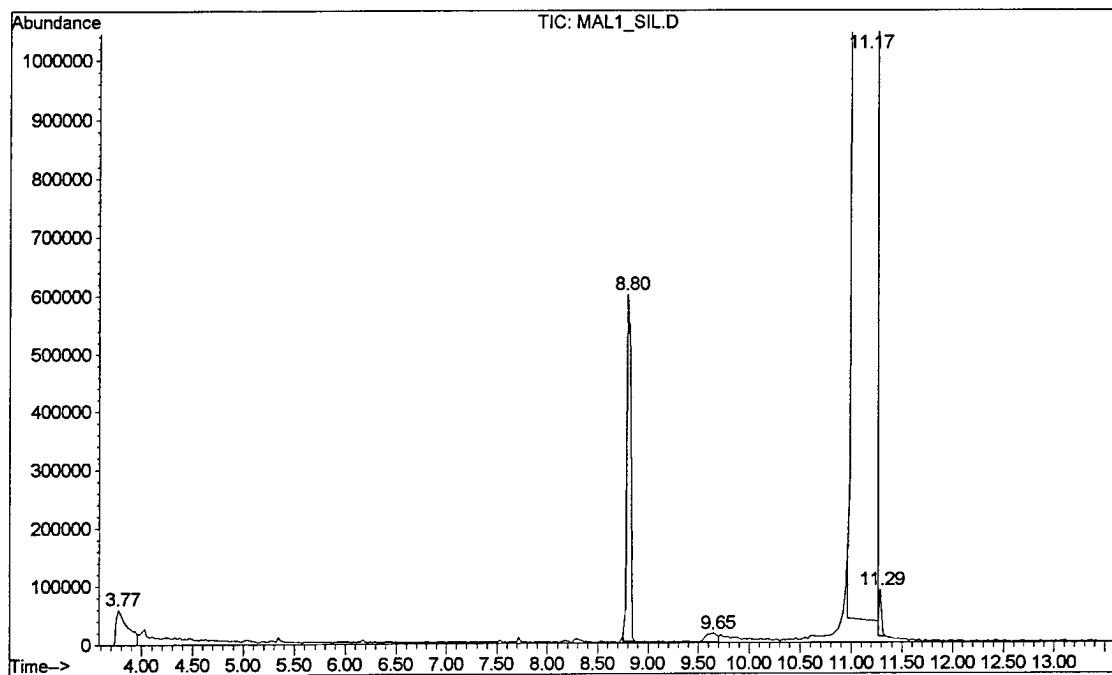
Regression Line for THR



Appendix C

GC-MS of Commercial Grade Malic Acid

File : D:\ELINK\INSTR1\METHODS\MAL1_SIL.D
Operator : geo
Acquired : 29 Aug 2001 16:31 using AcqMethod METHOD.M
Instrument : Finnigan
Sample Name: 1000ppm Malic Acid
Misc Info : Mal#1 Purity Check 400ul ea. BSTFA/Pyr. to 4ml2.0 ul inj.
Vial Number: 0



File : D:\ELINK\INSTR1\METHODS\MAL1_SIL.D
Operator : geo
Acquired : 29 Aug 2001 16:31 using AcqMethod METHOD.M
Instrument : Finnigan
Sample Name: 1000ppm Malic Acid
Misc Info : Mal#1 Purity Check 400ul ea. BSTFA/Pyr. to 4ml2.0 ul inj.
Vial Number: 0

